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Metabotropic Pathways Involved in the Generation of an Afterdepolarization in Layer V Pyramidal Neurons

Shannon Michele Linton

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

University of Washington
2000

Department of Physiology and Biophysics

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Abstract

Metabotropic Pathways Involved in the Generation of an Afterdepolarization in Layer V Pyramidal Neurons

Shannon Michele Linton

Chairperson of the Supervisory Committee
Professor Wayne E. Crill
Physiology and Biophysics

A previous study from our laboratory found that evoked spikes were followed by a long lasting afterdepolarization (ADP) in neocortical pyramidal neurons when metabotropic glutamate receptor (mGluR) agonists were present in the bathing solution. The Na^+ and K^+ currents involved in ADP generation depended on Ca^{2+} influx through voltage-gated channels and a rise in intracellular Ca^{2+} concentration. The present experiments were aimed at determining the mGluR subtype(s) and intracellular events underlying the ADP, as well as whether the ADP could be evoked with physiologic stimulation. Layer 5 neurons in neocortical slices from Sprague-Dawley rats (aged 14-24 days) were visualized with IR/DIC optics and recorded from using somatic whole-cell patch techniques. Drugs were introduced intracellularly via the patch pipette and in the bath. Experiments employing GDP-β-S confirmed that the mGluR-mediated ADP is dependent upon the activation of a G-protein. Experiments using specific pharmacological mGluR agonists and antagonists determined that the mGluR-mediated ADP was due to the activation of both Group I mGluR subtypes (mGluR1 and mGluR5). Activation of Group II mGluRs had no effect on the afterpotential. Both heparin and ryanodine significantly

reduced the ADP amplitude, indicating the dependence of the ADP on release of intracellular Ca^{2+} from both IP_3-sensitive and Ca^{2+}-sensitive stores. Biochemical studies have shown that intracellular IP_3 levels evoked by activation of Group I mGluRs are significantly elevated by concurrent activation of Group II mGluRs. We found that stimulation of Group II receptors using the specific agonist DCG-IV alone did not cause an ADP, but the amplitude of the Group I (DHPG)-mediated ADP was significantly potentiated by the concurrent activation of the Group II receptors. These neurons also generate a spike-dependent, heparin-sensitive ADP when exposed to carbachol. However, no amplification of the carbachol-evoked ADP was observed with concurrent Group II activation, indicating that a specific interaction between Group I and Group II mGluRs is required for the amplification of the mGluR-dependent ADP. These experiments revealed the electrophysiological correlates in layer 5 pyramidal neurons of several mGluR-mediated events reported in biochemical studies.

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I special thank you as well to Wayne, Peter, and Albert Berger for serving as my reading committee and sharing the benefit of their years of experience as excellent scientific writers and editors.

DEDICATION

To My Husband Dean

First of all, I dedicate this final effort to you. You are the consummate example of a true companion in life – the best husband and friend a woman could wish for. You are loving, honest, and kind - able to withstand the stressful distractions that surround a wife pursuing not only a graduate and medical degree, but motherhood as well. You not only offered your steadfast support, but also generously demonstrated it in multiple ways that are immeasurable. You spent the many hours alone while I studied and worked toward my goals, and endured all of this gracefully. You insisted on balance in our lives, and that our love remain the priority. This was ultimately the glue that held it all together and kept both my perspective and our mutual goals intact. You have earned this degree as well. I cannot thank you enough for all that you have done for me. I love you and share this accomplishment with you.

To My Daughter Alexandra

My sweet baby, you may decide to read this many years from now to "see what Mama was doing" during all those long hours away at the lab and in my office... You came to us near the end of this endeavor, but were certainly an important factor as we maneuvered to bring it to closure! You are one of the primary reasons I keep my expectations high, as well as the motivation to resolutely bring my goals to fruition. You should know that the pride of my accomplishment pales in comparison to the joy I find in sharing my life with you.

To My Mother Nancy, and My Family

Dear Mom, I cannot find words to express my appreciation for your incredible gift of being Alexandra's "Nana" when we needed you most. Thank you for your consistent love and support for me over the years. My wonderful family, your lives were directly and indirectly affected as well by this pursuit. I humbly offer this dedication in appreciation of your patience, love, and encouragement over the years.

CHAPTER I: Review of Literature

A Different Type of Glutamate Receptor

Glutamate is the principal excitatory neurotransmitter in the brain. Until recently, glutamate was believed to act only via the ligand-gated opening of cation channels. Glutamate binds to receptors, opens ion channels and produces rapid, large conductance changes in the postsynaptic neuron. Due to this association with ion channels, these glutamate receptors were termed "ionotropic". However, in the late 80's researchers began to report that glutamate could also stimulate inositol phospholipid metabolism through the interaction with GTP-binding regulatory proteins, leading to the formation of inositol 1,4,5-trisphosphate (IP_3) and subsequent mobilization of intracellular Ca^{2+} (Sladeczek et al. 1985; Sugiyama et al. 1987). This mechanism was relatively slow compared with the ionotropic response, produced metabolic changes in the postsynaptic cell, and was not linked to large, rapid changes in neuronal membrane conductances. Prior to this, neuromodulation of glutamatergic input to neurons was thought to rely on other well-known G-protein linked receptor pathways and second-messenger systems (e.g. serotonin, norpepinephrine, dopamine, acetylcholine). It now appeared that a different type of glutamate receptor could also mediate the same function. The term "metabotropic" was first used in context with glutamate receptors by Sugiyama et al in 1987.

The Metabotropic Glutamate Receptors

In 1991, two independent groups cloned the first metabotropic receptor subtype cDNA (mGluR1) (Houamed et al. 1991; Masu et al. 1991). Four more subtypes (mGluR2,3,4,5) were quickly identified within the next year (Abe et al. 1992; Tanabe et al. 1992). A total of eight mGluR subtypes (mGlu6 from Nakajima et al. 1993; mGlu7 from Okamoto et al. 1994; mGlu7 from Saugstad et al. 1994; and mGlu8 from Duvoisin et al. 1995), as well as splice variants of mGluR1, mGluR4, and mGluR5, have since been cloned (Conn and Pin 1997).

The mGluRs are unusual in that, although they possess a seven transmembrane domain typical of the structure of G-protein-coupled receptors (GPCRs), they also have a large N-terminal extracellular domain. Other GPCRs bind their ligand within the transmembrane domain region, but it is thought that glutamate binds to the N-terminal region in mGluRs. In 1993, a report stated that the sequence of this extracellular region was similar to bacterial periplasmic binding proteins (O'Hara et al. 1993). This information contributed to the hypothesis that the N-terminal was comprised of two "hinged" globular domains, between which the glutamate would bind. Recent studies have shown that this globular domain is indeed the site of mGluR agonist binding and subtype specificity, but it does not confer agonist potency. Another region, possibly downstream or in the carboxy terminal, may influence agonist/antagonist potency and specificity. (Schoepp et al. 1999). In GPCRs, the third intracellular loop was shown to be critical in coupling specificity with G-proteins (Strader et al. 1994). However, G-protein coupling and transduction mechanism specificity of the mGluR has been shown

to be located in the second intracellular loop (Gomeza et al. 1996). The N-terminal extracellular domain does not influence or modify the G-protein coupling specificity of the mGluR (Parmentier et al. 1998).

As the above might suggest, the mGluRs appear to have little in common with the remainder of the GPCR super-family, which prompted early speculation that they formed their "own" family of GPCRs. However, there is growing evidence that this may not be the case. A bovine parathyroid calcium-sensing receptor (CaR) is reported to have ~30% sequence identity with the mGluRs (Brown et al. 1993). This CaR is not sensitive to mGluR agonists but does respond to other cations such as Mg^{2+}, Gd^{3+}, and to polyvalent cations such as neomycin. Multiple mGluR subtypes have been shown to have calcium-sensing functions as well, with the calcium-sensitivity residing in a single amino acid residue in the N-terminal (Kubo et al. 1998). A more recent paper also reports similarity between a cloned $GABA_B$ ($GABA_B$ R1a) receptor and mGluRs (Kaupmann et al. 1997). Best-fit sequence alignments of the $GABA_B$ R1a with mGluRs revealed 18-23% amino-acid sequence identity and 43-48% related residues. Although the amino-acid sequence identity is low, the authors reported conservation of the structural architecture between $GABA_B$ R1a and the mGluRs based on hydrophobicity profiles. The $GABA_B$ R1a's sequence is not similar to $GABA_A$ receptors, $GABA_C$ receptors, or to G-protein-coupled receptors other than the mGluRs. The authors also report a structural similarity to bacterial periplasmic binding proteins evident in the N-terminal extracellular domain of $GABA_B$ R1a. With regard to further elucidation of mGluR function, the significance of these other GPCRs is unknown at this point, but strongly suggests that instead of being

unique to their own family, the mGluRs are part of a growing new family of G-protein coupled receptors. Further research in this area will more than likely enrich the understanding of the mGluR family as well.

Classification and Signal Transduction of mGluRs

Not long after the initial discovery of the new glutamate receptor, the presumption that all mGluRs would activate the IP_3 pathway was dispelled. As mentioned above, the subtypes began to be cloned and studied in various expression systems, and a significant difference became immediately clear with the discovery of mGluR2. The mGluRs 2,3,4,6,8 were coupled to the inhibition of adenylate cyclase and resulted in a decrease in the amount of cAMP produced from G_s stimulation. Only mGluR5 appeared to have a second-messenger pathway and pharmacological profile similar to the original mGluR1. Based primarily on extensive biochemical studies of native mGluRs in brain slices, primary neuronal or glial cultures, and clonal expression systems (for review see Conn and Pin 1997), it became clear that the family of mGluRs could be classified into groups based on amino acid similarity, transduction mechanism, and pharmacology. The mGluRs are separated into three main groups. The amino acid sequence within each group share ~ 70% sequence identity, whereas comparison between the groups show only ~ 45% similarity. Table 1 summarizes the characteristics of mGluR groups. Group I receptors (subtypes mGluR1,5) are coupled via $G_q/_{11}$ to the stimulation of phospholipase C (PLC) and subsequent phosphoinositide (PI) hydrolysis. The Group II receptors (subtypes mGluR2,3) and Group III receptors (mGluR4,6,7,8) are coupled via Gi/Go to

adenylyl cyclase and will inhibit the formation of cAMP evoked by the activation of an intrinsic Gs-coupled receptor. Stimulation of both Group II and III mGluRs is sensitive to pertussis toxin, implicating G_i involvement. There are mixed reports about the pertussis toxin sensitivity of Group I mGluRs.

Table 1 Families of metabotropic glutamate receptors and their associated receptor subtypes and mechanisms of action. (Conn and Pin 1997)

Receptor Families	Receptor Subtypes	Mechanism
Group I mGluRs	mGluR1 and mGluR5	Positively coupled via $G_{q/11}$ to PLC and PI hydrolysis
Group II mGluRs	mGluR2 and mGluR3	Negatively coupled by G_i/G_o to adenylyl cyclase to inhibit formation of cAMP evoked by activation of an intrinsic Gs-coupled receptor.
Group III mGluRs	mGluR 4,6,7,8	Same as Group II

Another transduction mechanism for mGluRs might exist. In 1993, it was reported that glutamate and metabotropic agonists could activate phospholipase D (PLD) in hippocampal slices of newborn and adult rats (Holler et al. 1993). Ionotropic glutamate receptor (iGluR) antagonists did not inhibit this response. Research has not yet elucidated the molecular identity of this mGluR subtype.

Pharmacology of mGluRs

In addition to sequence identity and transduction mechanisms, each of the mGluR groups has a characteristic pharmacological profile that assisted in their categorization. The specific ionotropic agonists (AMPA, NMDA, and kainate) are not active at any mGluRs, whereas quisqualate (AMPA / kainate agonist), ibotenate (NMDA agonist), and glutamate (the endogenous neurotransmitter) are active at all mGluRs. However, the different rank potency of these drugs (as well as others) within each group of mGluRs has been the foundation that scientists relied upon for years to explain which mGluR was involved in their study. Researchers recognized the need to identify reliable pharmacological tools to allow study of specific subtypes within each group. The initial development of specific pharmacological agents focused on phenylglycine derivatives. These agents were tested in environments with heterologous expression of mGluRs and ranged in activity from full agonist to competitive antagonist. It was determined that the antagonist activity was found in the (S)-enantiomers (Watkins and Collingridge 1994). The phenylglycine structure has provided a model from which the majority of the specific mGluR receptor ligands have been created and the list of specific mGluR subtype agonists and antagonists continue to grow each year.

A thorough review by Schoepp et al (1999) outlines in detail the potencies and subtype selectivities for mGluR drugs, the type of assays used, the species used to test the drug, and the associated literature where these studies were first published. Table 2 provides the complete chemical name for the abbreviations listed below. The most recent summary of rank order agonist potency states that Group I mGluRs are most potently

stimulated by quisqualate > ABHxD-I > 3,5-DHPG = Glu > DHPMP > 1S,3R-ACPD > L-CCG-1 > 3-HPG > CHPG > t-ADA. The summary of Group II mGluR rank order agonist potency includes: LY-379268 = LY389795 > LY354740 = L-F2CCG-I > DCG-IV = L-CCG-1 = trans-MCG-I = 2R, 4R-APDC = S-4MEGlu > cis-MCG-I > ABHxD-I > Glu = 2S,4S-4MG = 1S,3R-ACPD = 1S,3S-ACPD > L-CBG-I.

The pharmacology for Group III is not as well developed as for Groups I and II. There is a greater diversity of subtypes and few pharmacological tools available to study this group. Currently, it is accepted that Group III receptors display the following rank order of agonist potency: S-4-phosphono-2-aminobutyric acid (S-AP4) ≥ (RS)-PPG ≥ S-serine-O-phosphate (S-SOP) > (2S, 1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1) > S-glutamic acid (S-Glu) > 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD).

As mentioned before, another mGluR subtype was discovered that appeared to be coupled to phospholipase D (PLD) (Holler et al. 1993). In general the compounds that activate PLD in neuronal tissue include: quisqualate ≥ DCG-IV = L-CCG-I > 3,5-DHPG > 1S,3R-ACPD > CHPG > L-cysteine sulphinic acid ≥ glutamate. It is apparent that this novel mGluR responds to agonists of both Groups I and II. Interestingly, the Group III agonist L-AP4 is not active at these receptors.

Table 2. Abbreviations of mGluR agonists listed and their full chemical name.

Abbreviation	Chemical Name
ABHxD-I	(1S,2S,4S,5S)-2-aminobicyclo[2.1.1] hexane-2,5-dicaroxylic acid
1S,3R-ACPD	1S, 3R-aminocyclopentane-1, 3-dicarboxylic acid
1S,3S-ACPD	1S,3S-1-aminocyclopentane-1,3-dicarboxylic acid
t-ADA	trans-azetidine-2,4-dicarboxylic acid
S-AP4	S-4-phosphono-2-aminobutyric acid
2R,4R-APDC	2R, 4R-4 aminopyrrolidine-2,4-di-carboxylic acid
L-CBG-I	(2S,1'S,2'S)-2-(2-carboxycyclobutyl) glycine
L-CCG-I	(2S,1'S,2'S)-2-(carboxycyclopropyl) glycine
CHPG	2-chloro-5-hydroxyphenylglycine
DCG-IV	(2S,2'R,3'R)-2-(2',3'-dicarboxycyclo-propyl)glycine
3,5-DHPG	S-3,5-dihydroxyphenylglycine
DHPMP	amino-3,5-dihydroxyphenylmethyl-phosphinic acid
Glu	S-glutamic acid
3-HPG	S-3-hydroxyphenylglycine
LY354740	(1S,2S,5R,6S)-(+)-2-aminobi-cyclo[3.1.0]hexane-2,6-dicarboxylic acid
LY379268	(-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY389795	(-)-2-thia-4-aminobi-cyclo[3.1.0]hexane-4,6 dicarboxylic acid
L-F2CCG-I	(2S,1'S,2'S)-3'3'-difluoro-2-(carboxycyclopropyl)glycine
cis-MCG-I	(2S,1'S,2'R,3'R)-2-)2-carboxy-3-methoxymethylcyclopropyl)glycine
trans-MCG-I	(2S, 1'S,2'R,3'S)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine
S-4MEGlu	S-4-methylenegluatmic acid
2S, 4S-4MG	2S, 4S-4-methylglutamic acid
S-SOP	(RS)-PPG ≥ S-serine-O-phosphate

Expression of mGluR Subtypes in the Brain

Early studies initially measured excitatory amino acid-stimulated PI hydrolysis from various regions of the brain. PI hydrolysis induced by mGluR agonists was first reported in the striatum, and later in various regions including cortex, hippocampus, and cerebellum (Schoepp and Conn 1993). The cloning of the mGluR family allowed *in situ* mRNA studies to be performed and subtype-specific antibodies to be developed for immunocytochemical labeling. This, in addition to the growing repertoire of available pharmacological tools, allowed a wealth of research into the distribution of the mGluR subtypes in the brain and their developmental expression. The review here will focus on the findings for mGluR1, mGluR2, and mGluR5.

In situ studies specifically localized mGluR1 mRNA to neurons and was distributed widely throughout the adult rat brain. Intensely labeled neurons were Purkinje cells of the cerebellum, olfactory bulb, and neurons in hippocampus, lateral septum, thalamus, globus pallidus, substantia nigra, and the dorsal cochlear nucleus. Moderately labeled neurons were seen in dentate gyrus, striatum, cingulate and entorhinal cortices, mammillary nuclei, red nucleus, and superior colliculus. In the developing rat brain, the level of mGluR1 expression gradually increased with age (Shigemoto et al. 1992). This increase was especially noted in the cerebellum, but did not increase notably in the cortex and corpus striatum (Casabona et al. 1997). The pattern of mGluR1 immunoreactivity closely correlated with that of mGLuR1 mRNA. Immunolabeling with specific antibodies occurred in the olfactory bulb, stratum oriens of CA1 and polymorph layer of dentate gyrus in hippocampus, globus pallidus, thalamus, substantia nigra, superior colliculus,

and cerebellum of the rat. Lower levels of mGluR1 alpha were present within neocortex, striatum, amygdala, hypothalamus, and medulla. Dendrites, spines, and neuronal cell bodies contained mGluR1. The mGluR1 was not detectable in presynaptic terminals (Martin et al. 1992).

Expression of mGluR2 mRNA was observed in Golgi cells of cerebellum, granule cells of the dentate gyrus, most neuronal cells of the cerebral cortex, and small neuronal cells (probably intrinsic neurons) of the main and accessory olfactory bulbs (Tanabe et al. 1992). Using a monoclonal antibody to mGluR2 and immunocytochemical methods, researchers reported intense mGluR2 immunoreactivity in neuropil of cerebral cortical regions, hippocampus, olfactory bulb, some diencephalic nuclei, dorsal cochlear nucleus, and cerebellar cortex. In the cerebellar cortex, mGluR2 immunoreactivity was seen only in Golgi cells. In Ammon's horn, mGluR2 immunoreactivity was marked in the stratum lucidum of CA3 and the stratum lacunosum-moleculare of CA1-CA3, but not detected in the stratum pyramidale. The authors found that mGluR2 was located not only presynaptically but also postsynaptically (Neki et al. 1996).

Abe et al (1992) reported mGluR5 mRNA expression in olfactory bulb, anterior olfactory nuclei, olfactory tubercle, cerebral cortex, hippocampus, lateral septum, striatum, accumbens nucleus, cortical regions of the inferior colliculus, and spinal trigeminal nuclei. In the cerebellum, only a small population of Golgi cells expressed mGluR5 mRNA. The expression level of mGluR5 was greater in cerebral cortex, hippocampus, corpus striatum, olfactory bulb, cerebellum and hypothalamus in postnatal rats and declined with age. The expression was very low or absent in the adult cerebellum

and hypothalamus (Casabona et al. 1997). Immunohistochemical methods have verified the above findings, as well as provided more differential detail at the cellular level. For instance, mGluR5 immunoreactivity was observed in the cell bodies and dendrites of striatum and cerebellum. In the hippocampus, dendritic fields were intensely immunoreactive while staining of pyramidal and granule cell layers was absent (Shigemoto et al. 1993).

The mGluR subtypes are obviously differentially distributed in the brain. For example, mGluR1 mRNA is concentrated in neuronal cells of the dentate gyrus and areas CA2-CA4 of the rat hippocampus (Shigemoto et al. 1992; Tanabe et al. 1992), while mGluR5 mRNA is specifically localized in pyramidal cells throughout areas CA1-CA4 of the hippocampus and granule cells of the dentate gyrus (Abe et al. 1992). There is mRNA encoding mGluR1 in Purkinje cells of the cerebellum, while mGluR5 is localized to only a small population of Golgi cells in this brain region. This strongly suggests that these receptors play unique functional roles in those areas of the brain. Another possible variable in determining the functional roles of the mGluRs are significant changes of mGluR subtype expression (in rats) at different ages. As noted above, there are marked differences between postnatal and adult rats. Even more detailed studies of the rat neocortex have shown expression of mGluR5 exclusively in layer V at postnatal days 3 and 7, but throughout the cortex by day 10 (Bevilacqua et al. 1995).

More recent publications using immunochemistry and electron microscopy have demonstrated segregation of mGluRs from iGluRs at synaptic junctions (Lujan et al. 1996; Lujan et al. 1997).. The mGluR1 and mGluR5 subtypes were localized in regions

described as the "perisynaptic annulus" in the cerebellum and hippocampus - with each subtype having a different gradient in expression moving distally from these perisynaptic regions. In contrast, mGluR2 was not closely associated with glutamatergic synapses in the dendritic plasma membrane. The authors propose that the unique distribution of each mGluR subtype may reflect requirements for different transduction and effector mechanisms between different domains of the same cell, and suggest that the precise placement of receptors is a crucial factor contributing to neuronal communication.

Physiologic Roles of mGluRs

Because of the subject of this thesis, the following review will focus on the physiologic roles of mGluR-mediated effects in postsynaptic neurons. The importance of mGluR activation in postsynaptic neurons is manifested by their broad influence on excitability. The regulation of the activity of a number of different types of ion channels, affecting intracellular calcium dynamics, and activating various intracellular messengers implicate the mGluRs in a number of important changes in postsynaptic neuronal physiology. These include depolarization, decreased spike frequency adaptation, switched mode of firing (from bursting to single spike), modified synaptic efficacy, epileptic foci and excitotoxic responses of neurotrauma or stroke (Schoepp et al. 1990; Desai et al. 1992; Zheng and Gallagher 1992; Schoepp and Conn 1993; Mukhin et al. 1996; Conn and Pin 1997; Kang et al. 1998; Merlin 1998; Strasser et al. 1998; Anwyl 1999). There is a large body of work concerning mGluRs in synaptic transmission, including both long-term potentiation and depression; the bulk of which addresses

mGluR subtypes expressed in the presynaptic membrane. The role of mGluRs in synaptic transmission is thoroughly described elsewhere (Anwyl 1991; Schoepp and Conn 1993; Conn and Pin 1997; Anwyl 1999) and will not be discussed further. The following review will briefly discuss mGluR regulation of ion channels and modulation of ligand-gated ion channels in neurons.

Regulation of Ion Channels

Metabotropic glutamate receptors have been shown to influence neuronal activity by modulating a number of different voltage-dependent and voltage-independent ion channels. The majority of mGluR-mediated modulation of ion channel activity results in an increase in neuronal excitability, but there are some reports of inhibitory roles as well (via stimulation/inhibition of inhibitory interneurons). Most research has focused on the ability of the mGluR to inhibit potassium channels, but they also inhibit voltage-gated calcium channels and activate a number of nonselective cation currents. This section will focus on some of the major ion channels influenced by mGluR activation. A recent review thoroughly covers this topic in significantly more detail (Anwyl 1999).

Potassium Channels

Numerous potassium conductances are modulated by mGluR activation. The majority of the findings to date describe inhibitory activity, although there are some reports of potassium channel activation (Anwyl 1999). Inhibition of potassium channel activity has direct effects on the excitability of neurons. Examples include inhibition of the leak conductance ($I_{K^+(leak)}$), calcium-dependent after-hyperpolarizing K^+ current

(I_{AHP}), slowly inactivating voltage-dependent current (I_M), and a slowly inactivating Ca^{2+}-dependent current (I_{ADP}).

The leak conductance is a potassium channel that is thought to be primarily responsible for setting the resting membrane potential (Vm) in neurons. Activation of mGluRs has been shown to inhibit this leak conductance, causing a depolarizing shift in the resting Vm, the appearance of an inward current (in combination with inhibition of I_M – see below) and an associated decrease in membrane conductance (McCormick and von Krosigk 1992; Guerineau et al. 1994; Takeshita et al. 1996; Schrader and Tasker 1997). This inward current reverses at the K^+ equilibrium potential and is voltage independent. G-protein involvement is necessary for inhibition of the leak conductance (Guerineau et al. 1994) and it appears to be mediated by Group I mGluRs (Takeshita et al. 1996; Schrader and Tasker 1997). One of the physiologic consequences of mGluR-mediated inhibition of the leak conductance is a change in firing behavior (from bursting mode to single spike mode) in both thalamic neurons (McCormick and von Krosigk 1992) and in neocortical neurons (Wang and McCormick 1993).

Neurons possess a calcium-dependent K^+ current that underlies the major part of the slow AHP (I_{AHP}). The slow AHP is largely responsible for the property of spike frequency adaptation. The Group I mGluR agonists are particularly potent at blocking this K^+ conductance in multiple cell types throughout the brain (Charpak et al. 1990; Gereau and Conn 1995; Anwyl 1999), but Group II and III mGluR agonists have not been shown to affect the I_{AHP} (Gereau and Conn 1995). The slow AHP is also blocked by mGluR, resulting in a reduction in spike frequency adaptation (Charpak et al. 1990; Desai

et al. 1992). The mechanism of mGluR-mediated I_{AHP} inhibition is still unclear. It appears to be dependent on G-protein activation, but the exact intracellular messenger that mediates the inhibition of the channel is yet unknown. The IP_3 pathway seems to be involved in the hippocampus, and there does not appear to be a role for PKC or PKA (Abdul et al. 1996a). There is a requirement for a transient elevation in intracellular calcium, and subsequent involvement of protein tyrosine kinase (PTK) (Abdul et al. 1996b). Selective inhibitors of PTK reduced the inhibition of I_{AHP} by mGluR agonists. In fact, an increase in I_{AHP} was observed with application of these PTK inhibitors without mGluR agonists present, suggesting a tonic inhibitory influence by PTKs.

The I_M is a slowly inactivating voltage-dependent current that contributes to the outward rectification observed upon depolarization. It also plays a role in the AHP and therefore assists with spike frequency adaptation (Brown and Adams 1980; Brown 1988). Inhibition of the I_M by mGluR agonists has been demonstrated in the hippocampus (Charpak et al. 1990; Shirasaki et al. 1994) as well as other areas of the brain (Anwyl 1999). It appears to be mediated by Group I mGluRs (Ito et al. 1992).

In the presence of mGluR agonists (and muscarinic agonists as well), a slow afterdepolarization (ADP) is observed in olfactory cortex (Constanti and Bagetta 1991; Constanti and Libri 1992) and neocortex (Schwindt et al. 1988; Greene et al. 1992; Greene et al. 1994) after a large depolarizing stimulus. The following will focus only on the mGluR-mediated responses. In olfactory cortex, the current underlying a portion of the mGluR-mediated slow ADP is calcium-dependent, associated with a decrease in membrane conductance, is inhibited by tetraethylammonium (TEA) or

tetrabutylammonium (TBA), reduced by either elevating extracellular K^+ or clamping the cell close to the K^+ equilibrium potential, reduced by Cd^{2+}, insensitive to atropine, and not inhibited by tetrodotoxin (TTX), external Cs^+ (blocks hyperpolarization-activated cation current), or external Ba^{2+} (blocks voltage-gated K^+ currents). The researchers concluded that the mGluR agonist inhibited a novel calcium-dependent, very slow potassium current (I_{ADP}). The inhibition of this potassium current reduces spike frequency adaptation and generates a slow ADP with superimposed spike discharge for up to several minutes following the large depolarization. Even at slow firing rates, the individual slow ADPs can become additive due to the slow time course of decay. The mGluR-mediated inhibition (and subsequent slow ADP) is via Group I mGluRs (Libri et al. 1997).

In the neocortex, mGluR agonists produced a slow ADP after evoked spikes. However, there were some differences between the findings in the olfactory cortex and neocortex. Notably, the slow ADP in neocortex was not blocked by TEA, but it was (as in olfactory cortex) insensitive to Cs^+, external Ba^{2+}, TTX, and reduced with external Cd^{2+} or Ca^{2+}-free external solution. It also differed from the olfactory cortex in that it was blocked by intracellular Ca^{2+} chelation with BAPTA. The authors concluded that the mGluR-mediated slow ADP was probably due to the combined activation of a nonselective cation current (see below) and the inhibition of a calcium-dependent resting K^+ current – similar to the one described in the olfactory cortex. Based on mGluR agonist potency profiles, they also concluded that Group I mGluRs were the most likely receptors mediating this response (Greene et al. 1994).

Calcium Channels

Influencing intracellular calcium dynamics has functional implications in synaptic transmission, synaptic plasticity, repetitive firing, and somatic integration of neuronal input (Stefani et al. 1996). The majority of research on mGluR-mediated modulation of voltage gated calcium channels has demonstrated inhibition, although there are some reports of calcium channel potentiation (Rothe et al. 1994; Chavis et al. 1995a; Chavis et al. 1995b). Only the high-voltage calcium channels subtypes (N, L, and P/Q) seem to be affected. The bulk of the research in this area has shown inhibition of N-type (ω-conotoxin sensitive) calcium channels in numerous areas of the brain. This inhibition can be induced with agonists for mGluR Groups I, II, and III and requires pertussis-toxin sensitive G-protein activation (Anwyl 1999). Because the inhibition of N-type channels is rapid and reversible (Swartz and Bean 1992; Choi and Lovinger 1996) and the application of analogues or antagonists of intracellular messengers had little effect (Lester and Jahr 1990), the hypothesis of a membrane-delimited pathway was proposed. This was elegantly demonstrated with cell-attached patch recording techniques (Swartz and Bean 1992). Only recordings using whole-cell or outside-out mode patch recordings were able to demonstrate mGluR-mediated inhibition of N-type channels. The activation kinetics of the N-type channel was reported slowed as well (in the presence of mGluR agonists), suggesting a voltage-dependency of the block (Bean 1989; Swartz and Bean 1992; Choi and Lovinger 1996). This was confirmed with voltage-dependent relief of mGluR-mediated inhibition using depolarizing pre-pulses. Also, the depolarizing pre-pulses used to relieve the inhibition of the N-type channel facilitated the calcium current

by a greater amount when the mGluR agonists were present (Choi and Lovinger 1996). This may be due to removal of a tonic resting G-protein mediation inhibition of these calcium channels (Kasai 1991; Boland and Bean 1993; Netzer et al. 1994). The activation of protein kinase C (PKC), consistent with the activation of Group I mGluRs, may also play a role in removing the tonic inhibition of calcium channels by G-proteins (Swartz 1993), as well as a possible modulator of mGluR-mediated inhibition of calcium channels (Swartz et al. 1993).

The inhibition of L-type (dihydropyridine sensitive) calcium channels is considerably slower, consistent with the involvement of a diffusible intracellular second messenger, although research has yet to elucidate which one. Agonists for all three mGluR groups also inhibit the L-type calcium channels. An increase in intracellular calcium may play a role in L-type mGluR-mediated inhibition, either from release from intracellular stores or entry from the extracellular space. Chelation of intracellular calcium with BAPTA (Shen and Slaughter 1998) or substitution of Ba^{2+} for extracellular Ca^{2+} interferes with the mGluR-mediated inhibition (Sayer et al. 1992). However, Sayer et al (1992) found that BAPTA did not interfere with mGluR-mediated inhibition of L-type channels in dissociated neocortical neurons.

Specific Group II agonists as well as high potency non-specific mGluR agonists block the postsynaptic P/Q calcium channel subtypes (Choi and Lovinger 1996). One study has demonstrated inhibition of presynaptic P/Q calcium channels with a Group III specific agonist and found no effect with a Group II specific agonist (Takahashi et al. 1996).

Nonselective Cation Channels

In addition to the inhibition of potassium and calcium currents, mGluR stimulation also activates both Ca^{2+}-dependent and Ca^{2+}-independent nonselective cation currents. As mentioned above, activation of these currents often result in the appearance of a slow afterdepolarization (ADP), or in some cases a slow excitatory postsynaptic potential (EPSP). This physiologic response, in addition to the mGluR-mediated inhibitory modulation of potassium channels, can have a significant impact on neuronal excitability. In many cases, the slow ADPs are of sufficient amplitude to cross spike threshold and, in a positive feedback manner, result in repetitive firing of action potentials. The mechanisms underlying these nonselective cation currents are not well understood. The mGluR-activated non-specific cation current has been described in many neuronal types including areas CA1 and CA3 of hippocampus, thalamus dorsolateral septal nucleus, cerebellar Purkinje cells, and dorsal root ganglion (Anwyl 1999). The examples below will focus on some representative findings in the hippocampus, thalamus, cerebellum, and cortex.

An mGluR-mediated Ca^{2+}-dependent nonselective cation current was studied in area CA1 of the hippocampus (Crepel et al. 1994; Congar et al. 1997a). The authors reported a conductance that was dependent on G-protein activation, calcium dependent (completely suppressed by chelation of intracellular calcium with BAPTA), modulated by divalent cations, reversed polarity at a membrane potential consistent with a nonselective current, and also capable of being generated with high frequency synaptic stimulation in the presence of K^+ channel blockers, iGluR, and GABA receptor

antagonists. The activation of this current was mediated by Group I mGluRs (Congar et al. 1997a).

Recently, Partridge and Valenzuela (1999) reported data regarding the role of the different intracellular calcium stores in the mGluR-mediated activation of the Ca^{2+}-dependent nonselective cation current in CA1. They used various stimuli, including bath application of agonists and high frequency presynaptic stimulation. In elegant experiments designed to either potentiate the nonselective cation current or block potential stores, they determined that both IP_3 and Ca^{2+}-sensitive stores contributed to the activation of the calcium-dependent conductance. They also noted that transmembrane influx of extracellular Ca^{2+} participates with the expected IP_3-sensitive store-dependent $[Ca^{2+}]i$ response to mGluR agonist application. Using varied concentrations of the Group I specific antagonist AIDA, they concluded that the mGluR subtype mediating this response was most likely mGluR5. The authors discussed the possible contributing mechanisms of calcium-induced calcium release (CICR), the role of $[Ca^{2+}]i$ on the modulation of IP_3 and Ca^{2+}-sensitive calcium release, and the filling states of the intracellular calcium stores.

In area CA3 of the hippocampus, mGluR agonists activate both a Ca^{2+}-dependent (Caeser et al. 1993) and Ca^{2+}-independent (Guerineau et al. 1995) nonselective cation current. Caeser et al (1993) reported in CA3 neurons of cultured hippocampal slices a voltage-dependent current that was activated by depolarization in the presence of an mGluR agonist. It was dependent upon the influx of calcium, as it was depressed by reducing the extracellular Ca^{2+} concentration, as well as the application of external Co^{2+}

or Cd^{2+}. Using fluorescent imaging techniques, they showed that the amplitude of the cation current was correlated with the intracellular calcium concentration. The current reversed at around –47mV and was primarily composed of Na^{+} ions, but was also some K^{+} ions as well. Another study done in area CA3 of hippocampal slice cultures found a very similar current, but their results differed in some significant ways (Guerineau et al. 1995). First of all, the current was not abolished by chelation of intracellular Ca^{2+}, although it was reduced in the presence of extracellular Ba^{2+}, Cd^{2+}, and Mg^{2+}. The current was sensitive to changes in extracellular Na^{+} and K^{+} and had a reversal potential consistent with a nonselective cation current. It was blocked by TEA, but not by Cs^{+}. The authors suggested that activation of the nonselective cation current was mediated by Group I mGluRs. This was based on the agonist potency profile as well as antagonism by the Group I antagonist MCPG. Interestingly, the current was not influenced by GDP-β-S, GTP-γ-s, or pertussis toxin – suggesting a G-protein independent mechanism of mGluR-mediated activation of this cation current.

Further evidence of a G-protein independent mechanism of mGluR-mediated activation of a nonselective cationic current in CA3 was reported recently (Heuss et al. 1999). The researchers found that synaptic stimulation of mGluR1 in the postsynaptic neuron could concomitantly activate a G-protein dependent and independent pathway. An mGluR-mediated slow EPSC was described and reported due to activation of a nonselective cation conductance based on reversal potential. This EPSC was blocked by tyrosine kinase inhibitors and not influenced by the G-protein antagonists. The authors concluded that this pathway was G-protein independent. A GDP-βS block of the mGluR-

mediated inhibition of the afterhyperpolarization current demonstrated the G-protein dependent pathway.

An mGluR-mediated slow EPSP in thalamic neurons (ventral posterior nucleus) appears to be due to the activation of a nonselective cation current (Golshani et al. 1998). This slow EPSP could be large enough to cross action potential threshold and be crowned by a tonic barrage of action potentials. Determining the nonselective cationic nature of the underlying current was based on the extrapolated reversal potentials of the corresponding EPSC in voltage clamp under different conditions. The current could be elicited from either high frequency stimulation of corticothalamic fibers (in the presence of ionotropic glutamate and GABA receptor antagonists) or local application of mGluR agonists. This current differed from previously described mGluR-mediated nonselective cation currents in that it was not reduced by the application of Cs^{+}, Ba^{2+}, and Cd^{2+}.

In the presence of ionotropic glutamate and GABA antagonists, synaptic stimulation of parallel fibers in the cerebellum evokes slow EPSPs in Purkinje cells that are mediated by mGluR1 (Batchelor et al. 1994). A recent report used microfluorometric recordings to describe an mGluR-mediated increase in intradendritic $[Na^{+}]_i$ (Knopfel et al. 2000). The mGluR1 EPSC as well as the increase in $[Na+]_i$ were inhibited by the mGluR antagonist S-MCPG. In the presence of ionotropic glutamate and GABA antagonists and TTX, bath application of the selective mGluR agonist 3,5-DHPG induced an elevation in $[Na^{+}]i$ which extended over the whole dendritic field of the Purkinje cell. The most likely source of the Na^{+} is from an mGluR1-activated calcium-independent cation current with a high selectivity for Na^{+} against Ca^{2+}. The calcium-dependent

cationic current was thought to be less involved due to the fact that the mGluR1 EPSC is relatively resistant to Ca^{2+} buffering.

There is a muscarinic-mediated slow ADP in rat prefrontal cortex reportedly due to a voltage-dependent nonselective cation current (Haj-Dahmane and Andrade 1996; Haj-Dahmane and Andrade 1998; Haj-Dahmane and Andrade 1999), but there are very few reports of a well-described mGluR-mediated nonselective cation current in cortical neurons. A very recent study using whole-cell patch clamp recordings from layer II/III pyramidal neurons reported that quisqualate induced an inward current via mGluR activation (Chu and Hablitz 2000). Bath application of the nonselective mGluR antagonist, (R,S)-alpha-methyl-4-carboxyphenyglycine (MCPG, [200-500 μM]) reduced the current by 70%. GDP-β- S in the pipette solution markedly reduced (72%) the quisqualate-induced inward current. The current-voltage relation of the current was linear with a reversal potential near 0 mV suggesting involvement of nonselective cation channels.

Both a muscarinic and an mGluR-mediated slow ADP have been recorded from Layer V neurons of the neocortex (Schwindt et al. 1988; Greene et al. 1992; Greene et al. 1994). Their properties are very similar, and the original report of the muscarinic ADP suggested that it might be due to a Ca^{2+}-dependent nonselective cation current. Further study of the possible current underlying the mGluR-mediated slow ADP revealed that the spike-evoked ADP is dependent upon the influx and elevation of intracellular Ca^{2+}, is not blocked by extracellular Ba^{2+}, Cs^{+}, TEA, or TTX, is reduced by lowering the either the extracellular Na^{+} or K^{+} concentration, and is still observed at the reversal potential for

K^+. The authors concluded that mGluR activation resulted in both the inhibition of a Ca^{2+}-dependent resting K^+ conductance and the activation of a Ca^{2+}-dependent nonselective cation conductance. A more recent study from a different lab details a calcium-dependent nonselective cationic current that underlies a depolarizing afterpotential in neocortical pyramidal cells, but this current was apparently not sensitive to an mGluR antagonist (Kang et al. 1998). However, the afterpotential described had significantly different properties than the one reported by Greene et al (1994).

Ion Channels of Synaptic Transmission

As mentioned previously, there is a great deal of research surrounding the role of mGluRs in synaptic plasticity. Conclusions from publications to date about the exact role of mGluRs in long and short term potentiation (LTP and STP) and long term depression (LTD) in various areas of the brain continue to raise contradictions and are consequently not easily summarized. The overall intent of this thesis is to focus on mGluR-mediated effects in the postsynaptic neuron; for that reason this section will briefly describe only a few examples of the transient/reversible effects that mGluRs have on the postsynaptic ionotropic glutamate receptor (NMDA and AMPA) responses.

Metabotropic agonists potentiate individual excitatory responses from exogenously applied AMPA and NMDA in visual cortex and dorsal horn spinal neurons (Wang and Daw 1996; Ugolini et al. 1997). Interestingly, activation of mGluRs in area CA1 of the hippocampus has been shown to enhance only the depolarizing/inward current response from exogenous NMDA application. There was no observable mGluR-mediated modulation of the AMPA response in the same neurons, making this an

apparently selective process. (Aniksztejn et al. 1991). Using specific pharmacological tools, the NMDA modulation in hippocampus was attributed to activation of Group I mGluRs (Fitzjohn et al. 1996) and reported to be mediated by PKC (Aniksztejn et al. 1992). Similarly, Group I mGluRs (Ugolini et al. 1997) and PKC (Wang and Daw 1996) were shown to be involved in the modulation of both the NMDA and AMPA responses in spinal motor neurons and visual cortex. Reports to date have indicated that postsynaptic modulation is mediated by Group I mGluRs and no contribution from Group II mGluRs. Those mGluR subtypes appear to serve as modulators of presynaptic activity/efficacy. An opposite mGluR-mediated effect on NMDA currents has been reported as well (Yu et al. 1997). These researchers report that specific activation of both mGluR1 and mGluR5 in cortical neurons attenuates the NMDA currents in a membrane-delimited mechanism. However, research is still needed to explain the differing reports of mGluR-mediated potentiation and attenuation of ionotropic currents.

The example above of selective mGluR modulation in CA1 neurons from the hippocampus is supported by a recent study using transgenic mice (Jia et al. 1998). The mice were bred to be mGluR5-deficient, but were reported to develop normally. These mice showed a complete loss of the NMDA-receptor-mediated component of LTP, but normal LTP of the AMPA-receptor-mediated component. This selective loss of the NMDA component was seen in three different genotypic backgrounds. They also demonstrated that the NMDA component could be rescued by stimulating PKC in the postsynaptic neuron. These results suggest that PKC may couple the postsynaptic

mGluR5 to the NMDA-receptor potentiation during LTP, and that this signaling mechanism is distinct from the AMPA-receptor component.

This story of selective modulation by an mGluR subtype and differing reports in the literature about mGluR-mediated effects is representative of the diverse capabilities of the mGluR family. In this case, the endogenous neurotransmitter glutamate – the same excitatory agonist at all involved receptors (NMDA, AMPA, and mGlu) is able to communicate to the postsynaptic cell in multiple concurrent ways and affect the function of that cell uniquely. The physiologic conditions that arise in vivo that require unique responses from the same neurotransmitter are not well understood. The story of synaptic communication and mGluRs is still unfolding and as mentioned, the topics of presynaptic effects and multiple forms of long term plasticity will not be discussed here. For the general purposes of this thesis, it is important to recognize that mGluRs contribute an important role in synaptic transmission and plasticity, and that they continue to provide intriguing mechanistic possibilities with the varied expression and developmental changes of molecular subtypes, capabilities of G-protein independent and dependent modulation of ion channels, activation of various protein kinases, production of multiple second-messengers, and manipulation of the intracellular calcium dynamics.

CHAPTER II: Introduction to Present Research

Previous Findings from Our Laboratory

Previous studies in our lab found that evoked spikes in neocortical Layer V pyramidal neurons were followed by a long lasting afterdepolarization (ADP) when muscarinic agonists were present in the bathing solution (Schwindt et al. 1988). A similar finding was reported a few years later with metabotropic agonists (Greene et al. 1994). Blocking ionotropic glutamate channels (with 2mM kynurenic acid) and muscarinic receptors (with 5 μM atropine) while stimulating mGluRs with various agonists isolated the newly discovered metabotropic response, although the ADP was observed as well without the other antagonists present (*per communication with P. Schwindt*). Only an afterhyperpolarization was present after evoked spikes in the control perfusate (Figure 1 and 2A), but evoked spikes in the presence of mGluR agonists in the perfusate revealed a slow ADP (Figure 1). The agonists used in these experiments were not specific for a particular mGluR Group or subtypes. ACPD is an agonist at both Group I and Group II mGluRs. In order to investigate which mGluRs were mediating the ADP, dose response experiments were done to establish agonist potency profiles (see previous Pharmacology section). The conclusion was that Group I mGluRs, most likely mGluR5 were involved. The amplitude of the ADP was spike dependent and peaked at 5-10 spikes at 50-100 Hz (Figure 2C), but could be generated with as few as one spike (Figure 2B). The application of mGluR agonist caused a baseline depolarization (~ 4mV) and decreased the spike

frequency adaptation of the neuron (Figure 3B and 3C). In some cases, the ADP was of sufficient amplitude to cross spike threshold and elicit self-sustained repetitive firing (Figure 3B). The ADP depended on Ca^{2+} influx through voltage-gated channels (Figure 4) and was blocked by chelation of intracellular Ca^{2+} with BAPTA (not shown). No change in ADP amplitude was noted with the application of extracellular Ba^{2+}, Cs^{+}, or TTX. The amplitude was decreased by the reduction of extracellular Na^{+} in the TTX experiments. The data suggested that the currents underlying the ADP were a combination of a Ca^{2+}-mediated decrease in a resting K^{+} current and the activation of a Ca^{2+}-dependent nonselective cation current.

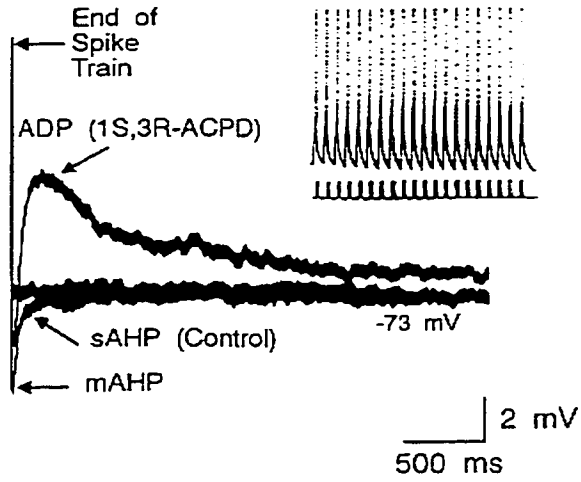

Figure 1. An afterdepolarization (ADP) follows a train of evoked spikes in the presence of mGluR agonist ACPD. Superimposed records of afterpotentials after a train of 20 spikes evoked at 100Hz by individual current pulses *(inset)* are shown in control perfusate and after agonist application. Example shown is from Greene et al (1994).

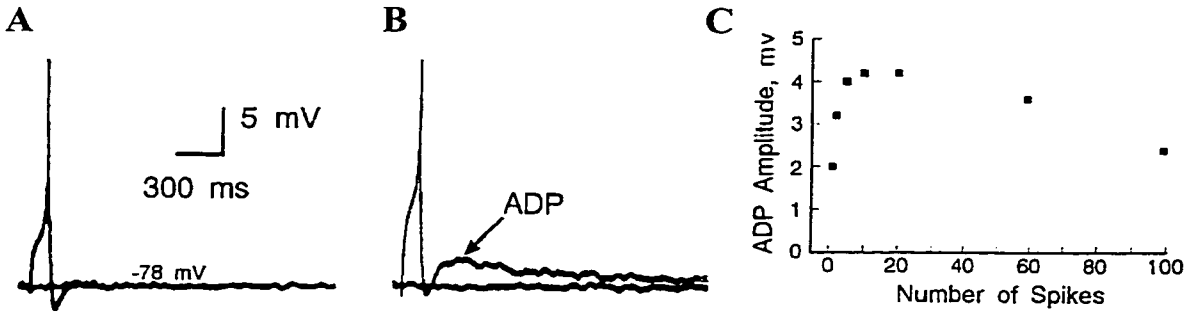

Figure 2. ADP amplitude increases with the number of preceding spikes Single action potential (*A*) was followed by a mAHP in control perfusate and by an ADP when 50μM ACPD was added (*B*). The plot of ADP amplitude vs. number of spikes evoked at 100 Hz by individual current pulses in a different cell (*C*). In all experiments perfusate also contained 2 mM kynurenic acid and 5μM atropine. Example shown is from Greene et al (1994).

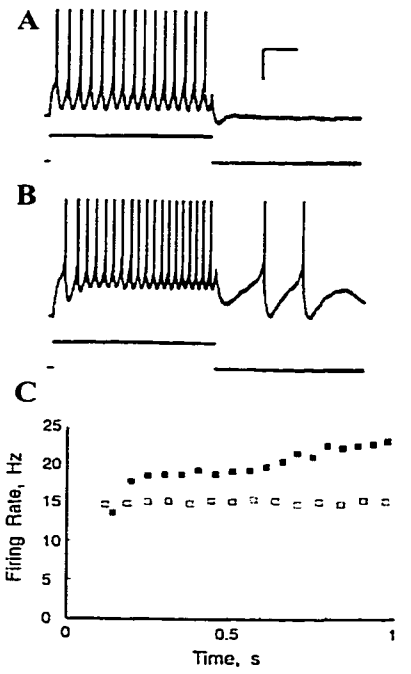

Figure 3. Alteration of repetitive firing by mGluR stimulation *A*: repetitive response (*top*) to injected current pulse (*bottom*) 1 sec in duration in normal perfusate. Action potentials are shown truncated. *B*: response evoked by the same current pulse after addition of 50μM ACPD to perfusate also containing 2 mM kynurenic acid and 5μM atropine. This figure also demonstrates an ADP of sufficient amplitude to cross spike threshold and generate repetitive action potentials. *C*: plot of instantaneous firing rate (1/interspike interval) vs. time for same cell in normal perfusate (*open squares*) and after ACPD (*closed*). Example shown is from Greene et al (1994).

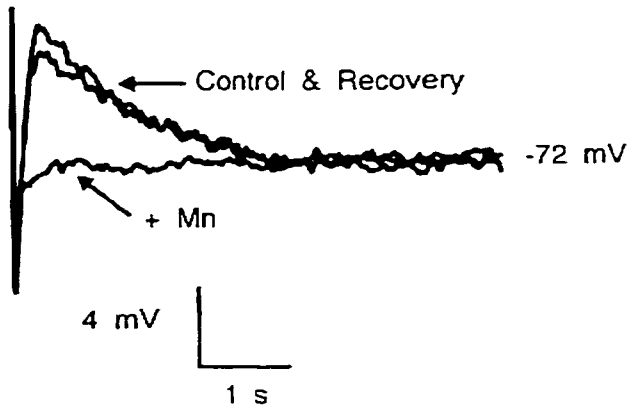

Figure 4. Blockade of Ca^{2+} influx abolishes ADP. Superimposed traces of afterpotentials after 20 spikes evoked at 100Hz in 50 μM ACPD. An ADP followed the spike train when the perfusate contained 2 mM $CaCl_2$ (control), but not after $CaCl_2$ was replaced with $MnCl_2$ (+ Mn). The ADP reappeared after reperfusion with Ca^{2+}-containing solution (recovery). Example shown is from Greene et al (1994).

Thesis Objectives

As described in the previous chapter, primarily biochemical studies and expression systems have determined the metabotropic glutamate receptor (mGluR) to be a G-protein-coupled receptor further classified by amino acid similarity and pharmacology. Our lab had studied a physiologic response to mGluR stimulation in neocortical cells and suggested possible ionic mechanisms underlying this response. However, we did not explore the intracellular biochemical events any further. The objective of the present research was to provide evidence of the physiologic consequence of intracellular biochemical events associated with mGluR activation in Layer V neocortical neurons.

Although convinced that I was observing an mGluR-mediated response, I needed to confirm that the ADP was dependent upon G-protein activation. The data collected previously in our lab suggested that the ADP response might be mediated by mGluR5, but this was based only on dose response curves and agonist potency profiles that were incapable of discriminating specific mGluR subtypes. The prior experiments had used nonspecific mGluR agonists. In particular, ACPD is nonspecific mGluR agonist at both Groups I and II. I needed to explore the possible contribution of both mGluR groups to the ADP. Fortunately, the pharmacological tools are now available to confirm the specific mGluR groups and receptor subtype(s) involved. We had established that the slow ADP was dependent upon an elevation in intracellular calcium, but the response also required the presence of extracellular calcium. Presumably there is requirement for both influx through ion channels (provided by the action potential and voltage gated Ca^{2+} channels) and release from intracellular stores. I was curious which intracellular calcium stores were being recruited with receptor activation. In addition to confirming the potential role of the well-known Group I pathway that activated IP_3-sensitive intracellular Ca^{2+} stores, was there a role for Ca^{2+}-induced Ca^{2+} release (CICR)? Finally, the mGluR-mediated slow ADP in neocortical neurons had only been observed using the application of pharmacological agents in the perfusate. For this response to be considered important physiologically, it was important to explore whether or not it was possible to elicit the mGluR-mediated ADP using synaptic stimulation and endogenous release of the neurotransmitter glutamate. The following chapters will describe these pursuits and discuss the significance of the results.

CHAPTER III: Methods

Tissue Preparation

Sprague-Dawley rats of either sex (14-24 days old) were anesthetized with an intraperitoneal injection of ketamine (150mg/kg) and xylazine (10mg/kg) and killed by carotid section. After removing the skull, the dorsal frontoparietal (sensorimotor) cortex was dissected away. The cortical block of tissue was fixed with cyanoacrylite glue to the stage of a microslicer (Vibratome) and then immediately submerged in ~ 4°C physiological saline solution (PSS) (see below) saturated with 95% oxygen / 5% carbon dioxide (carbogen). Transverse slices (300μm thick) were obtained and subsequently stored on mesh floats placed on the surface of carbogenated PSS at 34-35°C in a sealed holding chamber (partial interface environment). For experiments, the tissue slices were placed in a submerged chamber in carbogenated PSS flowing at a rate of 2-3 ml/minute at 32-34°C. A #1 glass coverslip formed the bottom of the 20 mm diameter, 3 mm deep recording chamber. The slices were held in place using a 300μm-thick U-shaped platinum wire with thin glass bars to straddle the slice. The gravity-fed inflow and suction outflow were positioned to promote laminar flow across the slice.

Recording

Recording pipettes were pulled from VWR 75μl micropipettes (extracellular pipette resistance 2-4MΩ) and filled with standard patch solution (see below). An upright

microscope (Zeiss Standard 16) fit with a 40X water immersion lens and DIC optics enabled viewing cells in the top ~75μm of the slice. Layer V neurons were visualized with IR/DIC optics, a CCD camera (Sony) and a high-resolution video monitor (Sony). Image resolution was enhanced using infrared illumination (Omega 770/40 bandpass filter). Visualized cells were recorded with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) used in bridge mode to observe somatic membrane potential and inject current through the recording pipette. Recorded potentials were corrected for a tip potential of 10 mV. Stable recordings lasting 1-2 h were frequently obtained. The seal resistance formed with the soma membrane was > 1 GΩ before break-in to the whole-cell configuration. Series resistance in the whole-cell configuration was monitored with a hyperpolarizing current pulse and maintaining bridge balance during the experiment. Recordings were discarded if the series resistance became > 50 MΩ. Only data from cells with a stable resting membrane potential more negative than –60mV, overshooting action potentials, and the ability to fire repetitively in response to 1-sec long depolarizing current pulses were recorded. Because the ADP amplitude is spike-dependent (Greene et al. 1994), a consistent stimulus throughout the experiment was necessary to observe drug effects. Trains of action potentials (1-20 spikes at 50-100 Hz) were elicited using a spike-train generator. The interval between spike trains (30s or 60s) was also controlled within each experiment by the use of a darkroom timer. The number of action potentials used to generate the mGluR-mediated ADP was chosen to maximize the response without eliciting a suprathreshold ADP that would evoke uncontrolled

repetitive firing. In most cases this was ~5-10 spikes. Synaptic stimulation was evoked by placing an extracellular bipolar electrode on the surface of the slice near the pia.

Analysis

Signals from the Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) were amplified, filtered at 10 kHz, and recorded on a multi-channel videocassette recorder with pulse code modulation (NeuroData, New York; 2 channel sampling rate 44 kHz). Resting potential was taken as the difference between extracellular and intracellular potentials monitored on a strip chart recorder. Recorded data were played back into a storage oscilloscope for analysis and photography or digitized with a 125 MHz digitizing board (Axon Industries TL-1 125) and analyzed using the DOS program WCP (John Dempster).

Solutions

The physiologic saline solution used both in tissue preparation and as a general perfusion solution was (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, and 20 glucose gassed with carbogen. The patch solution used in the electrodes was (in mM): 135 $KMeSO_4$, 5 KCl, 2 $MgCl_2$, 10 (3-[N-Morpholino] propanesulfonic acid) (MOPS-Acid), 0.1 Ethylene Glycol-Bis (β-Aminoethyl ether) n, N', N'-Tetraacetic acid (EGTA), 2 Na^+-ATP, and 0.2 GTP. In some experiments, additional drugs were added to the pipette solution to exert their effects intracellularly (see Table 3). No GTP or Na^+-ATP was used in the patch solution for experiments where GDP-β was also added

intracellularly. All experiments (except where noted) were done with [2mM] kynurenic acid and [5μM] atropine present in the bath. In addition to this, synaptic stimulation experiments were done with bicuculline [20μM], 2-hydoxysaclofen [500μM], and L-trans-2,4-PDC [300μM] in the bath.

Pharmacology

The following drugs were obtained from Tocris Cookson (Ballwin, MO): (1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid (1S,3R-ACPD), (RS)-2-chloro-5-hydroxy-phenylglycine (CHPG), (S)-4-carboxyphenylglycine [(S)-4CPG], (2S,2'R,3'R) - 2-(2'3'-dicarboxy-cyclopropyl) glycine (DCG-IV), (S)-3,5-dihydroxyphenylglycine (DHPG), methylphenylethylpyridine (MPEP), (E)-2-methyl-6-(2-phenylethenyl)pyridine (SIB-1893), and trans-4-Carboxy-L-proline / L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-2,4-PDC). Low molecular weight heparin, ryanodine, GDP-β-S, and 2-hydroxysaclofen were obtained from Calbiochem (La Jolla, CA). The following drugs were obtained from Sigma (St. Louis, MO): carbachol, bicuculline, kynurenic acid, and atropine. Drugs were introduced both intracellularly via the patch pipette and in the bath perfusate.

Isolation of the mGluR-mediated response was accomplished as follows. The ionotropic glutamate channels were blocked with [2mM] kynurenic acid in the presence of mGluR agonists. Also, due to the fact that a cholinergic-mediated (muscarinic) ADP is present in these cells (Schwindt et al. 1988), [5μM] atropine was added to the control perfusate in order to remove any possible contribution of this ADP to the mGluR-

mediated response, as might occur if the mGluR agonists excited cholinergic neurons that were presynaptic to the recorded cell. Table 3 describes the pharmacological tools used in these experiments and the associated abbreviations used hereafter in the text.

Table 3 Drugs used in experiments and their associated action. Abbreviations (see above for entire chemical name) are listed as used in the text. Listed ALPHABETICALLY.

Drug	Action
ACPD	Group I and II metabotropic agonist
Atropine	Muscarinic antagonist
Bicuculline	$GABA_A$ antagonist
Carbachol	Muscarinic agonist
CHPG	mGluR5 subtype agonist
DCG-IV	Group II metabotropic agonist, NMDA agonist
DHPG	Group I metabotropic agonist
GDP-β-S	Blocks binding of GTP to G-protein
Heparin	Blocks IP_3-sensitive intracellular Ca^{2+} stores
2-Hydroxysaclofen	$GABA_B$ antagonist
Kynurenic acid	Ionotropic glutamate receptor antagonist
L-trans-2,4-PDC	Inhibits glutamate uptake
MPEP	mGluR5 subtype antagonist
Ryanodine	Depletes Ca^{2+}-sensitive intracellular Ca^{2+} stores
S4-CPG	mGluR1 subtype antagonist
SIB-1893	mGluR5 subtype antagonist

CHAPTER IV: Results

Stability of the ADP Over Time

In these experiments several pharmacological agents expected to affect the biochemical pathway leading from mGluR stimulation to ADP were introduced into the cell by diffusion through the patch pipette. Because this diffusion and the associated alteration of the ADP often took many minutes, it was important to examine in control experiments whether ADP amplitude would remain stable in the continued presence of agonist for a comparable time period in the absence of these intracellular agents. In addition, there are reports of desensitization of mGluR-mediated responses in neurons with prolonged or repetitive bath application of mGluR agonists (Guerineau et al. 1997). In these experiments, after beginning perfusion of the slice with the mGluR agonist ([50μM] ACPD or [10μM] DHPG), a stimulus (5-10 individual current pulses at ~100 Hz) was chosen to maximize (without crossing spike threshold and causing uncontrolled repetitive firing) the initial ADP at the beginning of each experiment. In most cells, the same stimulus was then repeated at regular intervals (every 30 seconds) for as long as the cell remained healthy (in some cases up to 120 minutes). From these cells, four were chosen as representative controls because they contained the most data over time. The peak ADP values from five-minute blocks were pooled, averaged, normalized with respect to the initial control ADP, and then plotted. In some cells, there was only one ADP to be measured during a particular five-minute block, but these data were included

to provide continuity in the trend over time. As Figure 5 clearly demonstrates, the peak ADP amplitude is stable over time and resistant to desensitization.

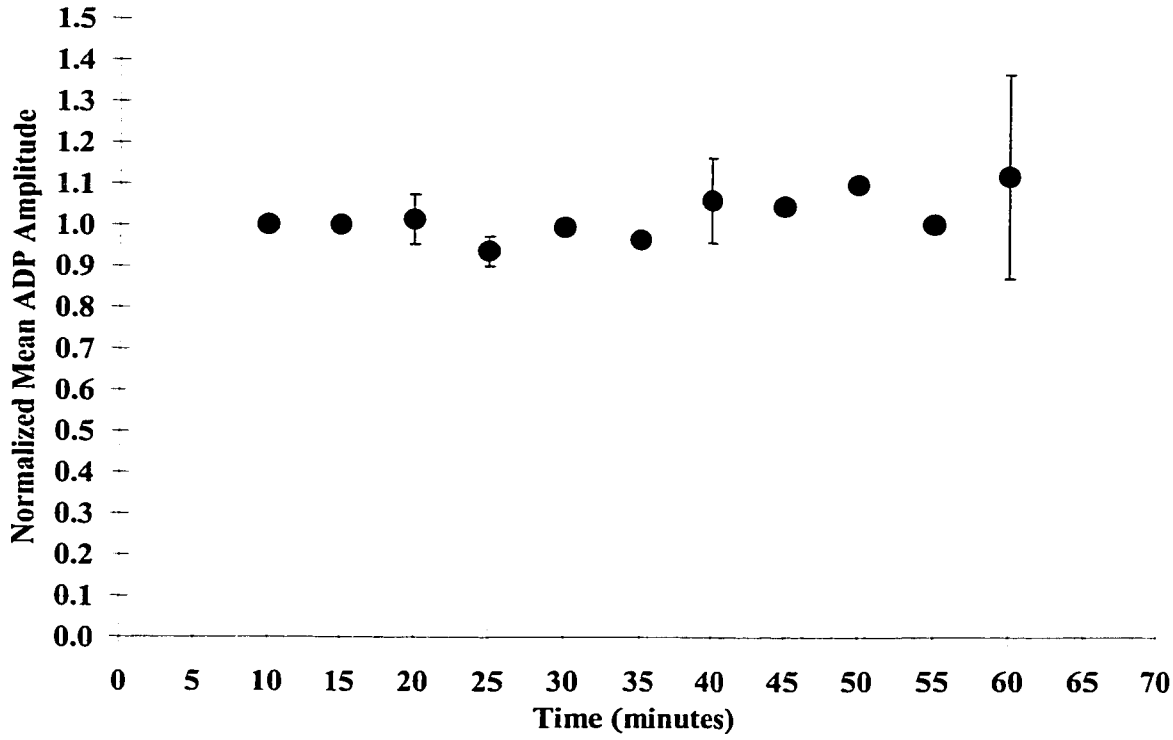

Figure 5. The mGluR-mediated ADP is stable over time. In the presence of continued mGluR agonist ([50μM] ACPD or [10μM] DHPG), ADPs were evoked at regular intervals over time. Plotted points (circles) are pooled (5-minute block) averages (+/- standard deviation of the mean) normalized to peak ADP amplitude at onset of each experiment from four representative cells. The ADP would remain stable in some recordings for up to 120 minutes (data not shown). Data points with no apparent error bar either have standard deviation that is not discernible beyond graphics or only have one data point for that 5-minute block (at 15,35,50,and 55 minutes).

G-protein Mediated ADP

Although by "definition", the metabotropic glutamate receptors are associated with G-proteins (Schoepp et al. 1990), there are reports of mGluR-mediated effects being G-protein independent (Guerineau et al. 1995; Heuss et al. 1999). To confirm that the physiologic response to mGluR activation in neocortical cells (the slow ADP) was dependent on an activated G-protein pathway, experiments were done to interfere with the G-protein mediated mechanism of mGluR activation. The drug GDP-β-S irreversibly binds to G-proteins and prevents GTP from binding (normally GTP is exchanged for GDP) when the associated receptor is activated. This disabling of the G-protein was expected to confirm the association between the specific agonist/receptor type chosen, the G-protein, and the ADP. GDP-β-S was introduced intracellularly via the patch pipette in the continued presence of the mGluR agonist ACPD [50μM]. To control for possible biochemical regenerative capabilities of GTP in the neuron, no GTP or Na^{+}-ATP was used in the patch solution for experiments when GDP-β-S was also added intracellularly. The amplitude of the ADP decreased over time as GDP-β-S diffused into the cell (n=4). The average decrease in ADP amplitude was 91% (+/- 0.06%) of control, with the ADP being completely abolished in one cell (see Figure 6). We are confident that this decrease in ADP amplitude is not caused by "run down" with receptor activation because, as shown in Fig. 5, ADPs in control cells were stable over the same duration. Thus, the mGluR-mediated ADP is dependent on G-protein activation.

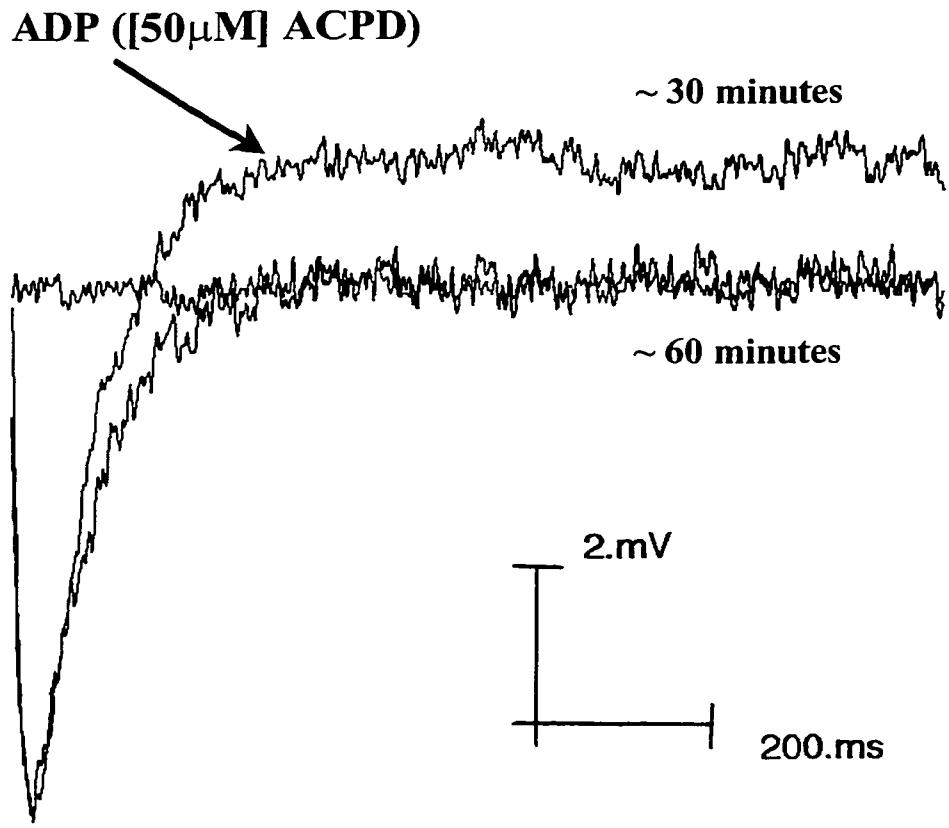

Figure 6. The mGluR-mediated ADP requires G-protein activation. GDP-β-S [1mM] in patch pipette with continued presence of mGluR agonist (ACPD) causes a decrease in ADP amplitude. Upper record from representative cell is at 30 minutes duration of whole cell access. Lower record recorded approximately 30 minutes later in same cell. Stimuli of 10 spikes evoked by individual current pulses at 100 Hz were given at regular intervals (every 60 seconds) to generate the mGluR-mediated ADP. Only the afterpotentials that followed the spike train are shown.

Intracellular Calcium Stores

The ADP in neocortical neurons requires an influx of Ca^{2+} thru VGCC and elevation in intracellular calcium concentration (Greene et al., 1994). Because the ADP is not observed without activation of mGluRs there is likely another source of mGluR-mediated calcium release that combines with the VGCC source and both are required for ADP generation. What is the source of calcium for the intracellular elevation? Would it be possible to demonstrate that the contribution of calcium from a particular source was integral to the production of an mGluR-mediated ADP? An obvious possibility of contribution to the required elevation in $[Ca^{2+}]_i$ for ADP generation is the Group I mGluR mechanism of generating IP_3 and the subsequent release of calcium from IP_3-sensitive intracellular stores. This had not yet been confirmed as playing a role in ADP generation in our cells. The previous experiments in our lab also could not rule out the possibility that after neocortical mGluRs are stimulated, some mechanism allows the Ca^{2+} influx to trigger the release of additional calcium from intracellular stores (calcium-induced calcium release (CICR)). There are reports of calcium, via influx through VGCC during spike trains, acting as a co-agonist at IP_3 receptors and stimulating additional calcium release from IP_3R-sensitive stores previously primed by IP_3 (Nakamura et al. 1999; Yamamoto et al. 2000). This mechanism was also consistent with the paradigm we were using to evoke an ADP. Additionally, if IP_3R-sensitive release of Ca^{2+} was involved, this might serve as a stimulus for additional CICR. The following experiments were designed to explore the role of the intracellular Ca^{2+} stores in the generation of the mGluR-mediated ADP.

IP_3-Receptor Sensitive Release

The activation of Group I mGluRs generates IP_3 and the consequent increase in $[Ca^{2+}]_i$ is believed to be due to the release of calcium from IP_3R-sensitive intracellular stores (Sladeczek et al. 1985; Sugiyama et al. 1987). The IP_3 receptor (IP_3R) is located on the endoplasmic reticulum of neurons and controls the opening of a specific channel and release of sequestered calcium ions into the intracellular milieu (Berridge and Irvine 1984). In order to determine if contribution from the IP_3R-sensitive calcium store was essential to the generation of the ADP, low molecular weight (LMW) heparin (5mg/ml) was added to the patch pipette solution and allowed to diffuse into the intracellular matrix after whole cell access. Heparin inhibits IP_3R channel opening by strongly competing at the IP_3 binding site (Ehrlich et al. 1994). The control mGluR-mediated ADP was evoked by spike trains in the presence of the mGluR agonist ACPD [50μM] as soon as possible after whole-cell access and stabilization of the cell. The stimulus (5-10 individual current pulses at ~100 Hz) was chosen to maximize the ADP. The same stimulus was then repeated every 30 seconds. The peak ADP values from five-minute blocks were pooled, averaged, normalized with respect to the initial control ADP, and then compared to cells where no LMW heparin had been introduced (Figure 7). The peak ADP amplitude was observed to decrease with time; presumably as more LMW heparin diffused into the cell and blocked the IP_3 receptors and release of calcium from this intracellular store. These results demonstrate that the Ca^{2+} from IP_3R-sensitive intracellular stores contributes to the required elevation of $[Ca^{2+}]_i$ for ADP generation. Estimate of the final effect of heparin on the amplitude of the ADP was taken from the asymptotic value of the exponential fit

to the data points in Figure 7. In eight cells, LMW heparin (5mg/ml) reduced the peak of the mGluR-mediated slow ADP by ~55%. These data demonstrate that the mGluR-mediated ADP in neocortical neurons is dependent upon the release of calcium from IP_3R-sensitive intracellular stores.

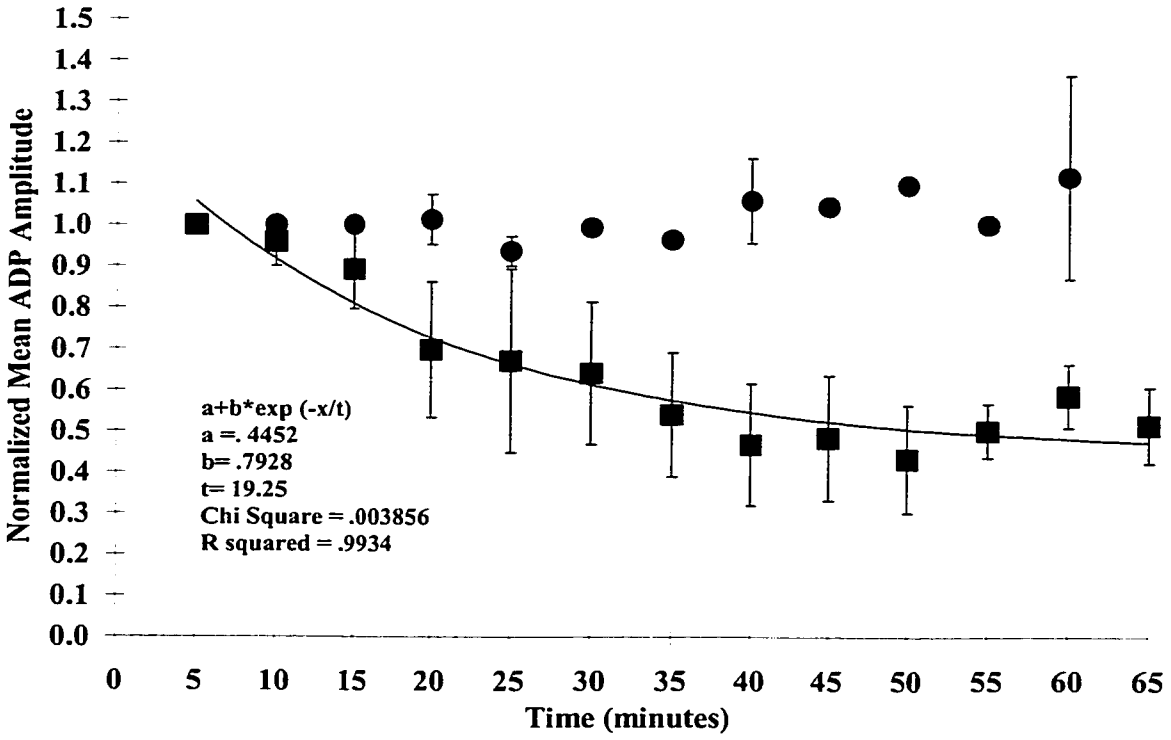

Figure 7. Low molecular weight heparin (5mg/ml) in pipette reduces the peak amplitude of the mGluR-mediated ADP over time. The mGluR agonist ACPD [50μM] was present continually in the bath. Plotted points (squares) are pooled (5 minute block) averages (+/- standard deviation of the mean) normalized to peak ADP amplitude at onset of experiment (n=8). *Inset*: Fit equation allows estimate of max effect from asymptotic value (decrease of ~55%). Control data (circles) from Figure 5 shown for comparison.

Ryanodine Receptor /Calcium-Sensitive Calcium Release

The entry of extracellular calcium via VGCC is also required to generate an mGluR-mediated slow ADP. Therefore, it is reasonable to suspect that elevation of intracellular calcium may stimulate the additional release of calcium from Ca^{2+}-sensitive stores (CICR) and contribute to the required elevation in $[Ca^{2+}]_i$ for ADP generation. Additionally, the release of calcium from IP_3R-sensitive stores may also stimulate CICR. Ryanodine binds to the ryanodine/caffeine-sensitive receptor on the endoplasmic reticulum that controls calcium-induced calcium release (CICR) from specific intracellular stores (McPherson et al. 1991). At low concentrations (<10μM), ryanodine binding is activity dependent, binding to the open channel and locking it permanently in an open subconductance state, thereby activated, but slowly depleting the intracellular (specific) calcium store (Rousseau et al. 1987; Smith et al. 1988). At higher concentrations, ryanodine still preferentially binds to the open state, but is inhibitory (Smith et al. 1988). In order to determine if CICR was essential to the generation of the ADP, ryanodine [50μM] was added to the patch pipette solution and allowed to diffuse into the intracellular matrix after whole-cell access. The control mGluR-mediated ADP was evoked by spike trains as soon as possible after whole-cell access and stabilization of the cell. The stimulus (5-10 individual current pulses at ~100 Hz, repeated every 30 seconds) was the same as used for the heparin experiments described above. As with the heparin experiments, the peak ADP values from five-minute blocks were pooled, averaged, normalized with respect to the initial control ADP, and then compared to cells where no ryanodine had been introduced (Figure 8). The peak ADP amplitude was

observed to decrease with time; presumably as more ryanodine diffused into the cell and blocked the ryanodine receptors and release of calcium from this intracellular store. Estimate of the final effect of ryanodine on the amplitude of the ADP was taken from the asymptotic value of the exponential fit. In six cells, [50μM] ryanodine reduced the peak of the mGluR-mediated slow ADP by ~49%. These results demonstrate that CICR from ryanodine-sensitive intracellular stores contributes to the required elevation of $[Ca^{2+}]_i$ for ADP generation.

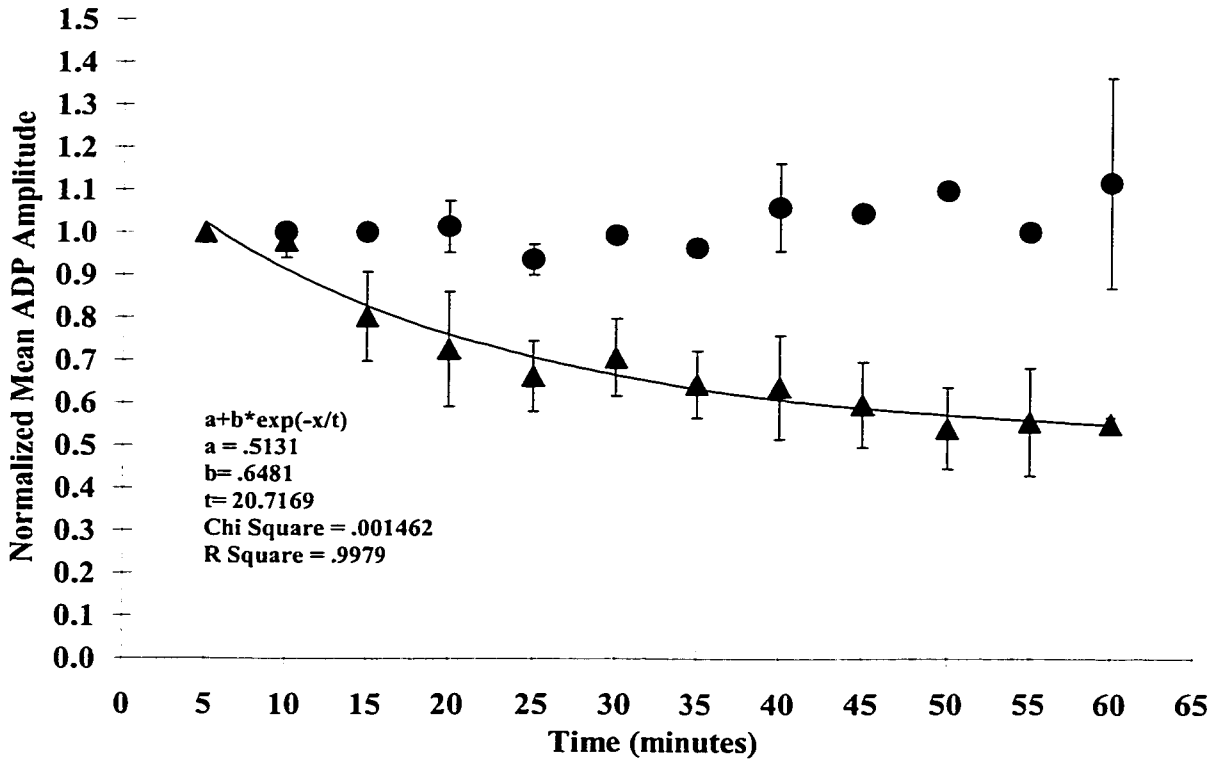

Figure 8. Ryanodine [50μM] in pipette reduces the peak amplitude of the mGluR-mediated ADP over time. The mGluR agonist ACPD [50μM] was present continually in the bath. Plotted points (triangles) are pooled (5 minute block) averages (+/- standard deviation of the mean) normalized to peak ADP amplitude at onset of experiment (n=6). *Inset*: Fit equation allows estimate of max effect from asymptotic value (decrease of ~49%). Control data (circles) from Figure 5 shown for comparison.

Contribution of Combined Stores

Data from my experiments confirmed the involvement of both heparin- and ryanodine-sensitive calcium stores in the generation of an mGluR-mediated ADP. The dosages of heparin and ryanodine chosen were significantly greater than most cited in the literature (1-2mg/ml for heparin; [20-40μM] for ryanodine). With each drug, a maximal effect had been achieved at these doses as indicated by the plateau reached during the exponential decline of the ADP amplitude. If combining the two drugs at these doses eliminated the ADP, it would demonstrate that contribution from both stores was necessary for ADP generation. In essence, the experiment would be testing whether a complete pharmacological block of Ca^{2+} release from intracellular calcium stores was similar to the experiment where the intracellular calcium was buffered with BAPTA (Greene et al. 1994).

The same experiment as previously described for heparin and ryanodine was repeated with both drugs in the pipette solution. Again, the peak ADP amplitude was observed to decrease with time; presumably as more of the IP_3 and ryanodine receptors were blocked and release of calcium from these intracellular stores was reduced, but the reduction did not plateau within the duration of our experiments as it had in the individual drug experiments (see Figure 9). The pooled, averaged, and normalized data of the combined drug experiment was fit with an exponential equation. The time constant (τ = 73.53 minutes) suggested that the two drugs combined did not influence the ADP amplitude as quickly as the two drugs individually (heparin τ = 19.25 min; ryanodine τ =

20.72 min). An estimate of the final effect of combined heparin and ryanodine on the amplitude of the ADP was taken from the extrapolated, asymptotic value of the exponential fit. In four cells, the extrapolated asymptote suggested that the ADP would have been reduced by ~94% by the combined LMW heparin (5mg/ml) plus [50μM] ryanodine if the recordings could have been maintained long enough. These data suggest that calcium release from both IP_3R-sensitive intracellular stores and CICR is required for the elevation of $[Ca^{2+}]_i$ necessary for mGluR-mediated ADP generation.

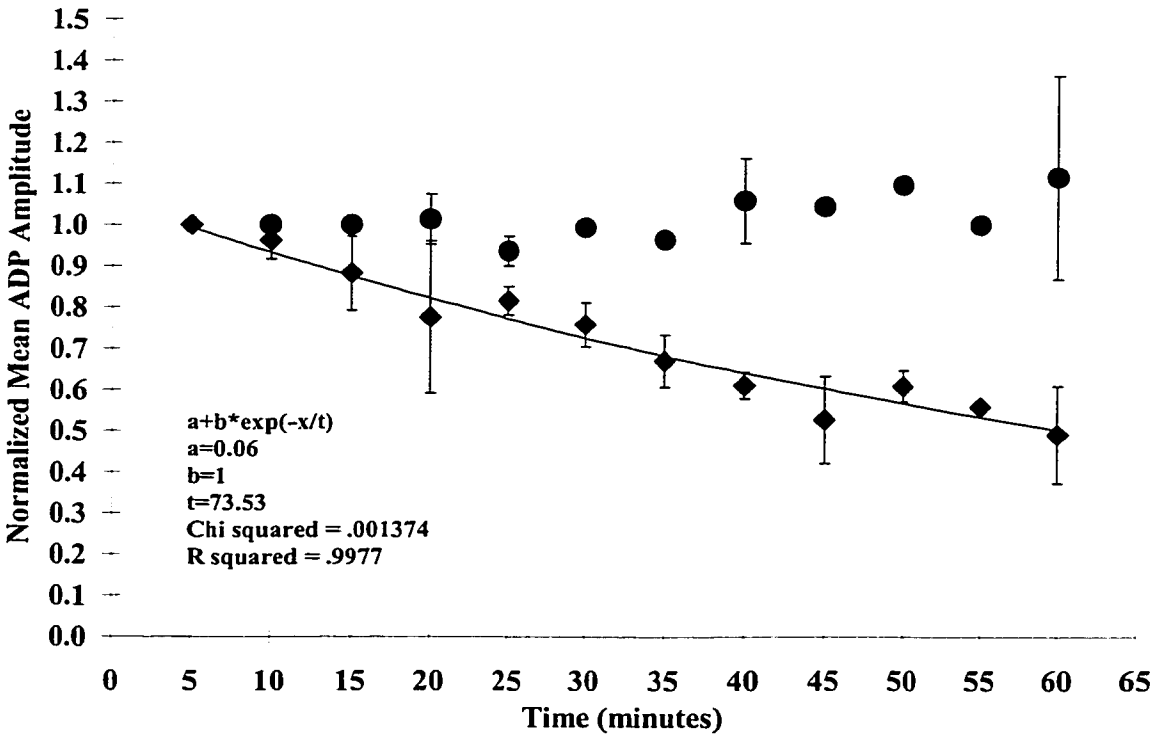

Figure 9. Combined LMW Heparin (5 mg/ml) and Ryanodine [50μM] in pipette reduces the peak amplitude of the mGluR-mediated ADP over time. The mGluR agonist ACPD [50μM] was present continually in the bath. Plotted points (diamonds) are pooled (5 minute block) averages (+/- standard deviation of the mean) normalized to peak ADP amplitude at onset of experiment (n=4). *Inset*: Fit equation allows estimate of maximum effect (decrease of ~94%). from extrapolated asymptotic value. Control data (circles) from Figure 5 shown for comparison.

Exploring the mGlu Receptor Type Mediating the ADP

Specific mGluR agonists were not available for previous experiments in our lab (Greene et al. 1994). I now had the pharmacological tools available to explore the responsible metabotropic receptor Group(s) underlying the slow ADP in neocortical neurons. The primary mGluR agonist used by Greene et al (1994) was ACPD. This drug activates both Group I and Group II receptor subtypes. Both receptor groups are well expressed in rat cerebral cortex (see previous discussion in Review of Literature); although within Group I, reports found only low levels of mGluR1 in neocortex, but significant expression of mGluR5. I was interested in isolating these two mGluR Groups to observe their relative contribution to the ADP response. Also, there were now pharmacological tools available to discriminate between the receptor subtypes mGluR1 and mGluR5 within Group I.

Group I Agonist Studies

Group I Agonist DHPG

Evoked spikes were followed by a long lasting ADP when the mGluR Group I agonist DHPG [10μM] was present in the bath (n=12). The average amplitude of the ADP with a 5-spike stimulus was 4.89 (+/-1.56) mV, similar to the previous ADPs evoked by 5 spikes in ACPD (Greene et al. 1994). In some cells, the ADP reached sufficient amplitude to cross spike threshold, resulting in repetitive spiking that required an injected hyperpolarizing current pulse to terminate (data not shown). My goal was to

generate a consistent maximal ADP that would remain sub-threshold for spike activation, as uncontrolled repetitive firing potentially disturbed the intracellular calcium dynamics and activation of mechanisms underlying the ADP. Greater concentrations of DHPG seemed to cause suprathreshold ADPs more frequently, so a lower concentration was chosen for the experiments. Figure 10 is a representative mGluR-mediated slow ADP using the agonist DHPG at [10μM]. Stimuli consisted of five individual current pulses to evoke spikes at 100 Hz. Stimuli were given at regular intervals, usually every 30 seconds. Data from 5-minute blocks were pooled and then averaged.

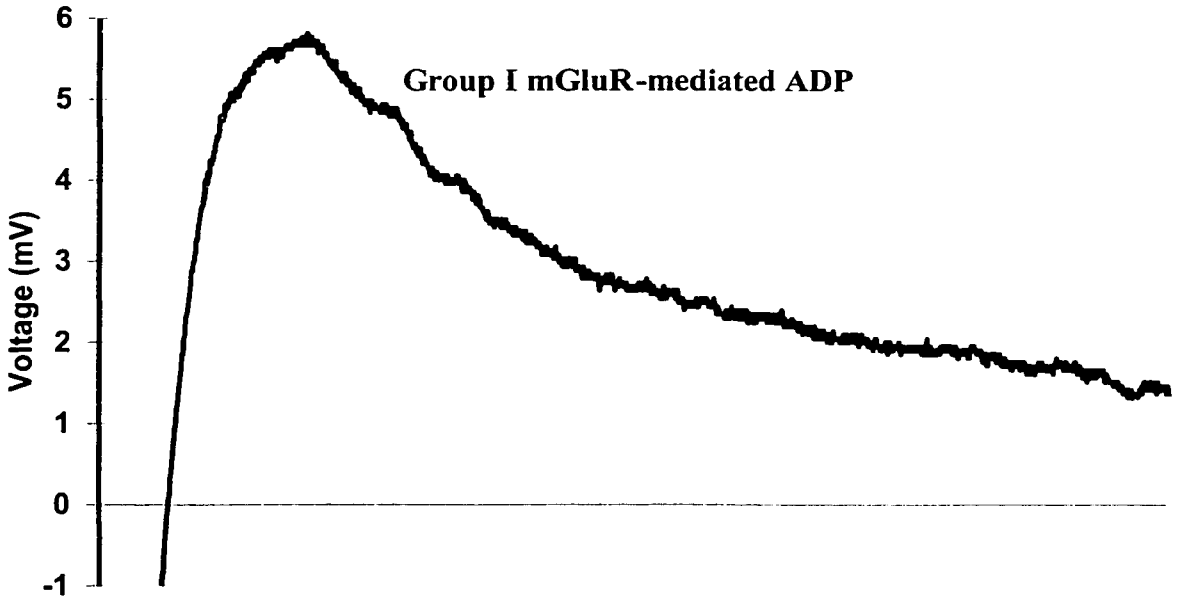

Figure 10 An ADP follows a train of five evoked spikes in the presence of mGluR Group I agonist DHPG [10μM]. Data is from one representative cell. Preceding 5-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Trace shown is an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

mGluR5 Subtype Agonist CHPG

I now had demonstrated that Group I mGluR activation was at least partially responsible for generation of the slow ADP in neocortical neurons. Group I mGluRs include the subtypes mGluR1 and mGluR5. As mentioned, the cerebral cortex expresses high levels of mGluR5. I tested the involvement of mGluR5 in ADP generation by using the specific mGluR5 subtype agonist CHPG. CHPG is a selective mGluR5 receptor agonist that is reported to be completely inactive at mGluR1 receptors (Doherty et al. 1997). This makes it the first agonist able to distinguish between these two subtypes. Further studies of the Group I subtypes mGluR1 and mGluR5 were carried out with specific mGluR subtype antagonists (next section). Using the same protocols as previous experiments, both [100μM] and [1mM] CHPG in the bath perfusate were able to generate a slow ADP following a spike-train stimulus in five cells (n=5). The average amplitude of the ADP was 1.9 (+/- 0.71) mV. Figure 11 is a representative mGluR-mediated slow ADP using the agonist CHPG at [1mM]. Stimuli consisted of five individual current pulses to evoke spikes at 100 Hz. Stimuli were given at regular intervals, usually every 30 seconds. Data from 5-minute blocks were pooled and then averaged. For clarity, the preceding 5-spike stimulus has been truncated from the plot. The x-axis is equivalent to approximately 4 seconds of sampled time.

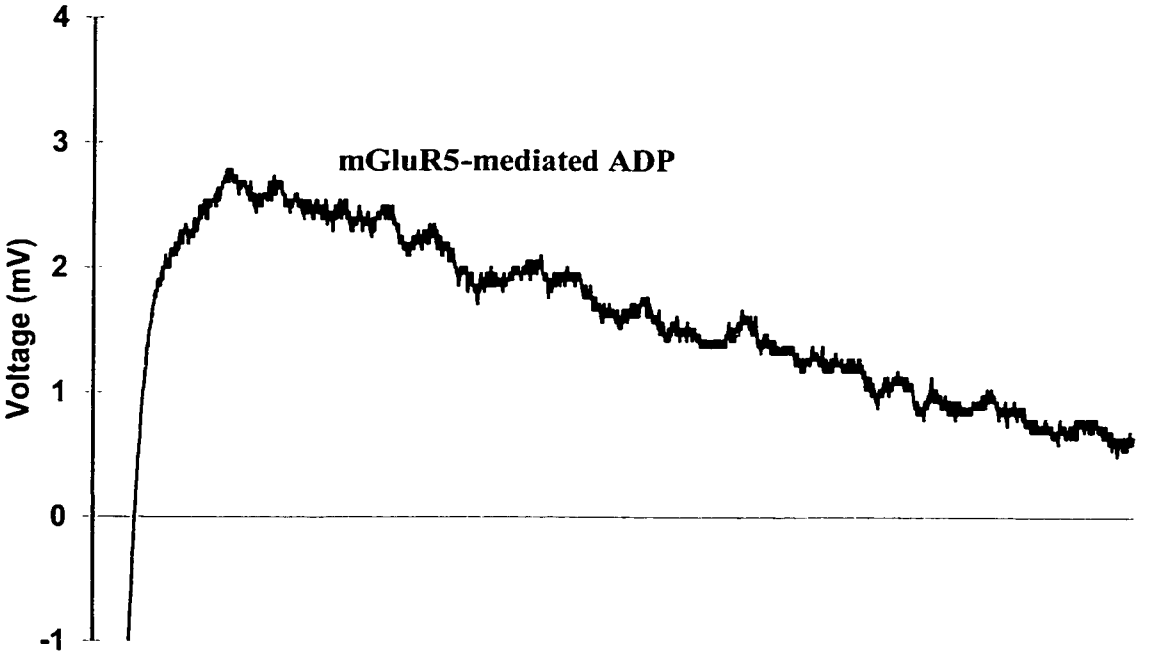

Figure 11. An ADP follows a train of five evoked spikes in the presence of the mGluR5 subtype specific agonist CHPG [1mM]. Data is from one representative cell. Preceding 5-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Trace shown is an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

Group I Antagonist Studies

The most specific Group I mGluR pharmacological tools currently available are not all agonists. I found that to pursue further study of the Group I mGluR-subtypes underlying the ADP, I would need to design experiments that would use mGluR-subtype-specific antagonists. This was necessary to confirm the involvement of mGluR5, as well as investigate the possible role of mGluR1.

mGluR5 Antagonist SIB-1893

SIB-1893 (E-2-methyl-6-(2-phenylethenyl)pyridine) is a highly selective mGlu5 receptor antagonist. It inhibits glutamates-stimulated calcium signals mediated by mGlu5 (IC_{50}=0.3μM), and is 100-fold less potent at inhibiting responses mediated by mGluRs1,2,4,6,7,8 (IC_{50}>30μM) (Varney et al. 1999). I decided to use this drug to confirm the contribution of mGluR5 activation in the Group I mGluR-mediated ADP. Using the Group I agonist DHPG [10μM] in the bath, ten spikes were evoked using individual current pulses at 100Hz. The stimulus was repeated regularly every 30 seconds. The slow ADP following these spikes (similar to Figure 10) was used as the control within each experiment. The control ADP was observed for 5-10 minutes to determine stability of the recording and response. Then the mGluR5 antagonist SIB-1893 [5-10μM] was added to the perfusate with the Group I agonist DHPG [10μM] still present. The results demonstrated a marked decline in ADP amplitude. The data from 5-minute blocks were pooled (~ 10 records) and averaged for comparison with other time blocks. The mGluR5 antagonist SIB-1893 caused an average decrease in ADP amplitude of 46.7 % (+/- 8.4 %) from the control value (n=3). The maximal effect of the drug was reached within 15-20 minutes. Figure 12 illustrates averaged traces (each trace is an average of ~10 records over a 5 minute period) from a representative cell. Displayed are the control ADP and maximal effect of the drug on the amplitude of the ADP (during the time block of 15-20 minutes). This data, in combination with my previous findings with the mGluR5 agonist CHPG, confirm that activation of the Group I mGluR5 contributes to the generation of the slow ADP in neocortical neurons.

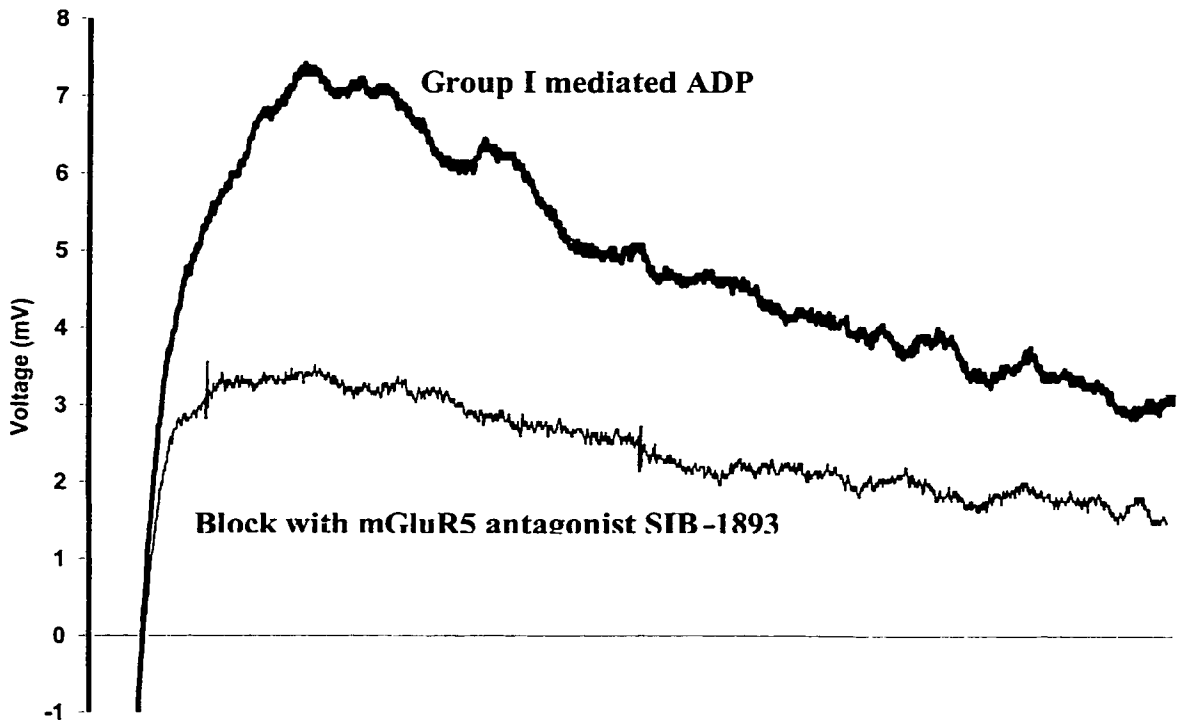

Figure 12. Block of a portion of the ADP by the Group I subtype mGluR5 specific antagonist SIB 1893 [10μM] indicates that mGluR5 activation contributes to ADP generation. *Top trace (dark line):* Control ADP using DHPG [10μM]. *Bottom Trace (light line):* ADP amplitude declined within 15 minutes of adding SIB-1893 [10μM] to the bath. Data is from one representative cell. Preceding 5-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Traces shown are each an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

mGluR5 Antagonist MPEP

MPEP (2-methyl-6-(phenylethynyl)-pyridine) is another new potent mGluR5 selective antagonist that has no effect on responses mediated by the other Group I subtype mGluR1. It also does not act as an agonist or antagonist at Group II (mGluR2,3) or Group III (mGluR 4,6,7,8) subtypes (Gasparini et al. 1999). I had already demonstrated that a mGluR5 subtype specific agonist (CHPG) was capable of generating

an ADP, as well as showing that a mGluR5 antagonist (SIB-1893) was capable of blocking a portion of a Group I mGluR-mediated ADP. MPEP was not only as selective, but more potent (IC_{50} = 43nM) when compared to SIB-1893. I was interested in observing the effect of this alternative pharmacological tool to block mGluR5 in my cells. Again, the control ADP was elicited using the Group I mGluR agonist DHPG [10μM]. The addition of MPEP [10μM] to the perfusate caused a significant decline in the ADP within 5 minutes by ~76% (n=1). This was a significantly greater than the decrease caused by SIB-1893. Figure 13 illustrates averaged traces from the cell. Displayed are the control ADP and maximal effect of the drug on the amplitude of the ADP (t=5-10 minutes). These data, in combination with my previous findings confirm that activation of the Group I mGluR5 contributes to the generation of the slow ADP in neocortical neurons.

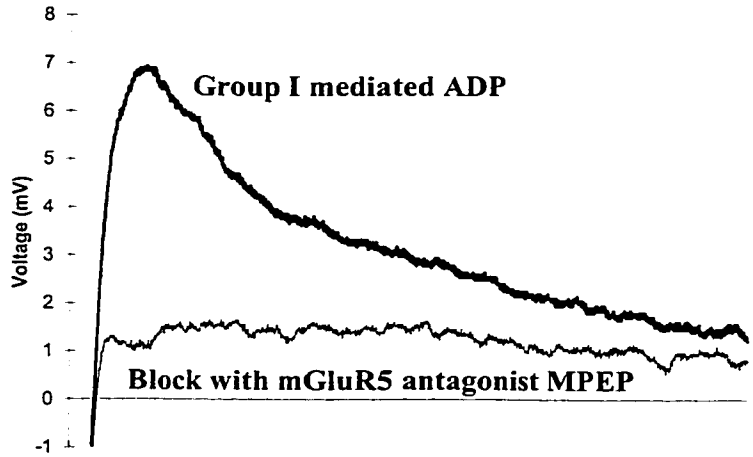

Figure 13. Block of a portion of the ADP by the Group I subtype mGluR5 specific antagonist MPEP [10μM] indicated that mGluR5 activation contributes to ADP generation. *Top trace (dark line)*: Control ADP using DHPG [10μM]. *Bottom Trace (light line):* ADP amplitude declined to 76% of control within 5 minutes of adding MPEP [10μM] to the bath. Data is from one representative cell.

mGluR1 Antagonist S4-CPG

Based on the data acquired to this point, the entire ADP could be mediated by only mGluR5. I did not do experiments to determine the maximum effect of the mGluR5 agonists and antagonists, as my primary goal was to simply demonstrate participation of the receptor group. The other Group I mGluR subtype also potentially activated by DHPG is mGluR1. Although studies have found limited expression of mGluR1 in cerebral cortex, my data had not yet excluded the possibility that the activation of mGluR1s could contribute to the release of calcium from intracellular stores necessary for ADP generation. There is a competitive Group I mGluR antagonist called (S)-4-carboxyphenylglycine (S4-CPG) selective for mGluR1 over mGluR5 (K_B= 84μM for mGluR1 using glutamate as an agonist (or 14.9μM using ACPD) versus >2000μM for mGluR5) (Brabet et al. 1995). This is currently one of the only pharmacological tools available to study mGluR1 contribution directly (versus finding maximal block of mGluR5 in Group I specific ADP and inferring that the remainder is mediated only by mGluR1). Using the same experimental protocol described above, I added S4-CPG [500μM] to the perfusate while stimulating Group I mGluR-mediated ADPs with DHPG [10μM]. The addition of the drug caused the amplitude of the ADP to decline. The mean decrease in amplitude was 57.5% (+/- 2.1%) from control (n=2). Maximal effect was achieved within 10-15 minutes. Figure 14 illustrates averaged traces (each trace an average of ~10 records over a 5 minute period) from a representative cell. Displayed are the control ADP and maximal effect of S4-CPG [500μM] on the amplitude of the ADP

(during the time block of 10-15 minutes). These data suggest that the mGluR-mediated ADP is also due in part to activation of the Group I mGluR subtype mGluR1.

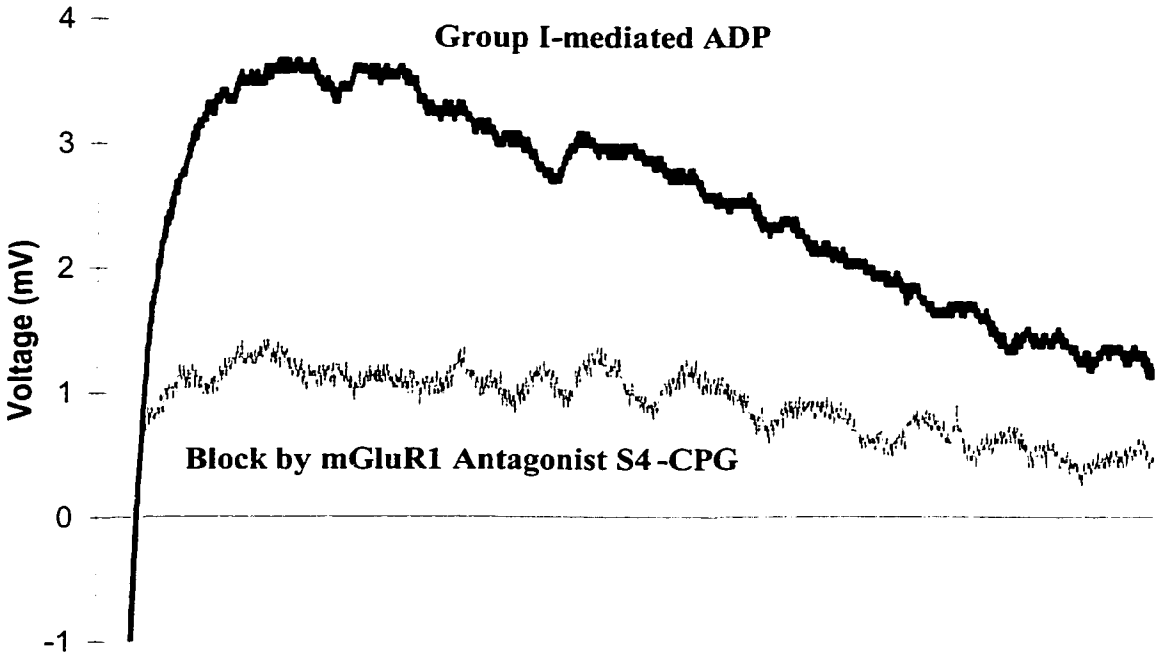

Figure 14. Block of a portion of the ADP by the Group I subtype mGluR1 specific antagonist S4 CPG [500μM] indicated that mGluR1 activation also contributes to ADP generation. *Top trace (dark line)*: Control ADP using DHPG [10μM]. *Bottom Trace (light line):* ADP amplitude declined within 5 minutes of adding S4-CPG [500μM] to the bath. Data is from one representative cell. Preceding 5-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Traces shown are each an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

Combined MPEP and S4-CPG

As I was using the specific Group I mGluR agonist DHPG to generate the control ADPs in the above antagonist experiments and had demonstrated that both mGluR1 and mGluR5 activation contributed to the mGluR-mediated ADP, I decided to see if the

combination of these two antagonists (at the doses chosen for the original experiments) would be sufficient to abolish the Group I mGluR-mediated ADP. Using the same experimental techniques described above, I added MPEP [10μM] and S4-CPG [500μM] to the perfusate while stimulating Group I mGluR-mediated ADPs with DHPG [10μM]. The addition of the drugs caused the amplitude of the ADP to decline. The mean decrease in amplitude was 86.5% (+/- 3.5%) from the control (n=2). Maximal effect was achieved within 5-10 minutes. Figure 15A illustrates averaged traces (each trace an average of records over a 5 minute period) from a representative cell. Displayed are the control ADP and maximal effect of the combined drugs on the amplitude of the ADP (during the time block of 5-10 minutes). The most likely reason for the ADP not being completely abolished is sub-maximal dosing of one or both of the antagonists. In one of the cells, both MPEP and S4-CPG were washed from the perfusate while the Group I agonist DHPG remained (Figure 15B). This resulted in a partial recovery of the ADP in ~10 minutes (potentially limited by the nearing the end of our recording time/stability capability with the cell) to ~59% of the control (from 16% of control). Although I was confident in the stability of the control ADP over time, this wash confirmed that the pharmacological agents mediated the observed decline in ADP amplitude.

I now had evidence from both specific agonist and antagonist studies to suggest that the mGluR-mediated ADP in neocortical neurons is mediated predominantly by the activation of both Group I subtypes mGluR1 and mGluR5.

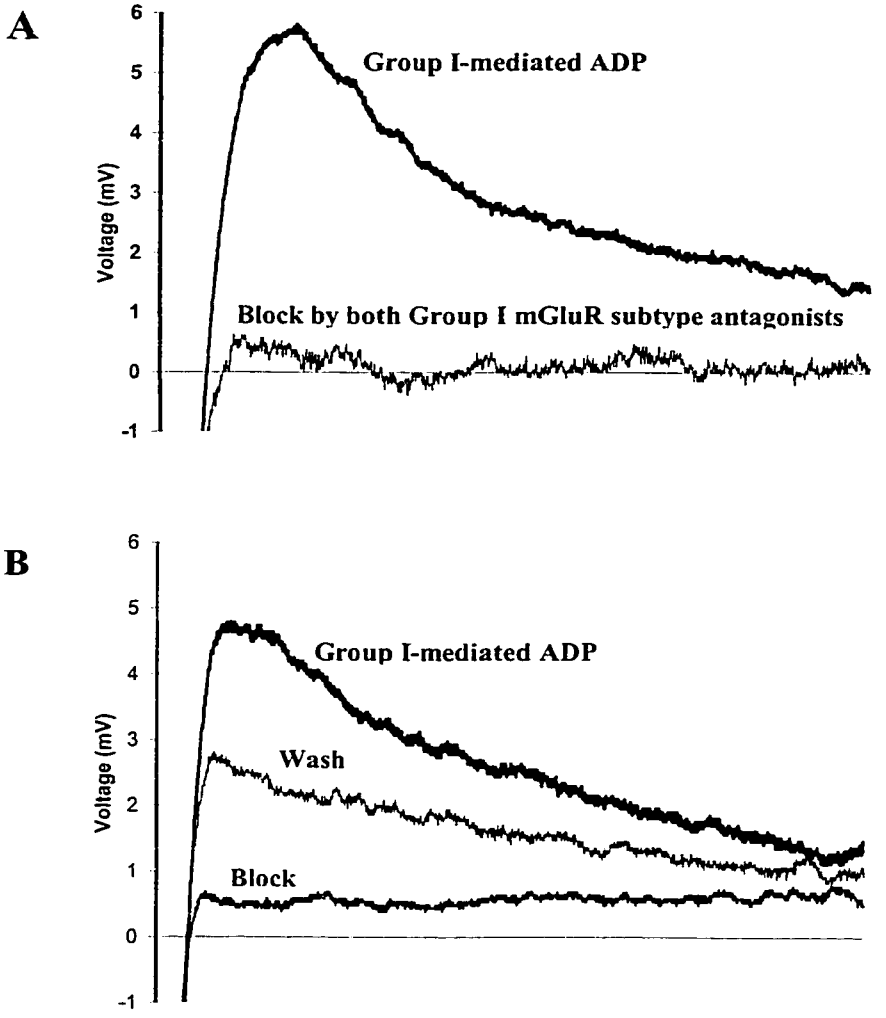

Figure 15. Block of a significant portion of the ADP by a combination of both the Group I subtype antagonists S4-CPG [500μM] and MPEP [10μM], indicating that both mGluR1 and mGluR5 activation contribute to ADP generation. A. *Top trace (dark line)*: Control ADP using DHPG [10μM]. *Bottom Trace (light line):* ADP amplitude declined to 89% of control within 10 minutes of adding S4-CPG [500μM] and MPEP [10μM] to the bath. **B.** *Top trace (heavy line)*: Control ADP using DHPG [10μM]. *Bottom Trace (medium line):* ADP amplitude declined to 84% of control within 10 minutes of adding S4-CPG [500μM] and MPEP [10μM] to the bath. *Middle Trace (light line):* In the continued presence of the agonist, wash of both antagonists from the perfusate results in a partial recovery of the ADP in 10-15 minutes. Further recovery was not possible due to termination of recording. *Both A and B:* Preceding 5-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Traces shown are each an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

Group II Agonist Studies

Previous experiments in our lab had used non-selective mGluR agonists to evoke the mGluR-mediated ADP (Greene et al. 1994). Importantly, the primary drug used to characterize the properties of the ADP (1S,3R-ACPD) was an agonist at both Group I and Group II mGluRs. The current experiments had demonstrated a dominant role for Group I mGluR activation, but it was necessary to evaluate the role of Group II mGluR activation in the generation of the ADP.

The drug DCG-IV (2S,2'R,3'R-2-(2'3'-dicarboxy-cyclopropyl) glycine) is a potent agonist at Group II mGluRs. It has been shown to significantly depress presynaptic release of glutamate (Ishida et al. 1993) and also have neuroprotective properties (Bruno et al. 1994). To test the response of my cells to specific Group II mGluR activation, I added DCG-IV [10μM] to the control perfusate and used the same stimulus protocol previously used to evoke mGluR-mediated ADPs. DCG-IV has also been shown to act as an agonist at NMDA receptors (Wilsch et al. 1994; Uyama et al. 1997). Because the normal control perfusate in my experiments contains kynurenic acid [2mM] to block ionotropic receptor activation (and atropine [5μM] to block muscarinic AChR activation) this property would be controlled for.

Evoked spikes were followed by a normal afterpotential when DCG-IV [10μM] was present in the bath (n=11). This was tested in a number of different ways. DCG-IV was applied as the first mGluR agent after obtaining records in the control perfusion solution (containing kynurenic acid and atropine). It was also observed as a single drug in

a "wash" after experiments with DCG-IV and other mGluR drugs were being performed (see next section). Figure 16 displays a representative cell from one of those experiments. Using a five-spike stimulus evoked by individual current pulses at 100Hz, a normal afterpotential is observed in the control perfusate. This same stimulus was then repeated with DCG-IV [10μM] in the bath. The slice was allowed exposure to DCG-IV for up to 15 minutes. There was no change in the afterpotential when compared with control. A baseline record is displayed for comparison as well. These data demonstrate that activation of Group II mGluRs alone does not contribute to the mGluR-mediated ADP observed in neocortical neurons.

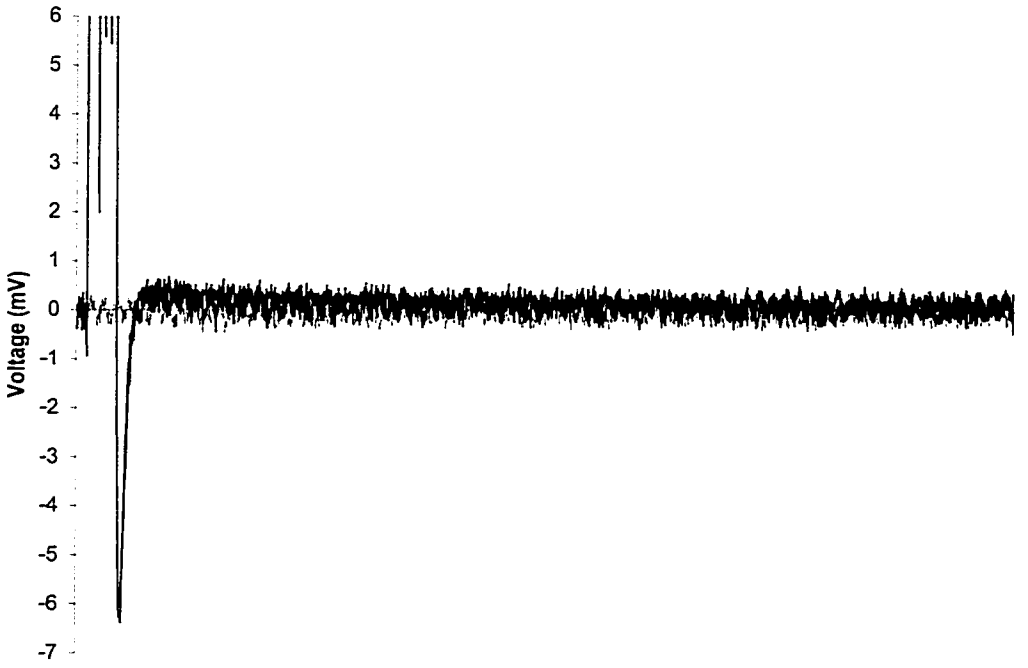

Figure 16. The Group II mGluR agonist DCG-IV [10μM] does not change the afterpotential following a spike train. The five-spike stimulus was evoked in control perfusate and then in the presence of DCG-IV [10μM]. No observable difference between the two conditions occurred. A control baseline record was included for comparison. Each trace shown is an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

Potentiation of the ADP

A number of papers in the literature describe an interaction between Group I and Group II mGluRs. Particularly relevant for the present research, reports using specific biochemical assays had found that the levels of IP_3 generated by Group I mGluR activation were amplified when the Group II mGluRs were concurrently activated. The following will briefly review some of these findings.

As early as 1993, when DCG-IV became available as a Group II agonist, reports appeared describing the enhancement of Group I stimulated phosphoinositol (PPI) hydrolysis with concurrent activation of Group II mGluRs. Utilizing biochemical assays of slices pre-labeled with myo-D-[^{3}H]inositol, researchers examined the effects of DCG-IV on the PPI hydrolysis evoked by quisqualate, ACPD, and muscarinic AChR agonists in various regions of the brain. Nicoletti et al (1993) reported that DCG-IV enhanced the PPI hydrolysis response of quisqualate, but not ACPD or carbamylcholine (mAChR agonist) in hippocampal slices.

From the same lab the following year, this work was refined by demonstrating an increase in IP_3 formation with concurrent ACPD and DCG-IV stimulation, but only at sub-maximal ACPD concentrations (Genazzani et al. 1994). The interpretation was that DCG-IV increased the potency of ACPD, but not the efficacy. They concluded that DCG-IV amplified the downstream activity of mGluRs only when the receptor population was partially activated, but had no impact on fully activated mGluRs. No increase in basal level of IP_3 was found in either hippocampus or cortex with DCG-IV exposure, but the increase was greater in the hippocampus than cortex in quisqualate-

mediated PPI hydrolysis with concurrent Group I/Group II exposure. By demonstrating the lack of effect of the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) when DCG-IV was present, the authors suggested that the mechanism of enhanced PPI hydrolysis involved a PKC-mediated phosphorylation process.

The effect of a different Group II mGluR agonist (2R,4R-APDC) on PPI hydrolysis stimulated by the specific Group I mGluR agonist DHPG was studied in hippocampus (Schoepp et al. 1996). They reported no increase in basal PPI hydrolysis with exposure to 2R,4R-APDC, but it greatly enhanced the PPI hydrolysis response to DHPG and quisqualate. Again, like the previously mentioned studies, they found that this Group II activation appeared to increase the potency of the Group I agonist, while not affecting the maximum response of the drug.

Perhaps even more significant for my purpose, a recent study used similar biochemical assay techniques to study mGluR-mediated IP_3 generation in the neonatal rat cortex (Schaffhauser et al. 1997). They first observed that the Group I mGluR agonist DHPG stimulated the production of lower levels of IP_3 than the Group I/II mGluR agonist ACPD. Using a potent Group II mGluR agonist (LY354740), they demonstrated that concurrent Group II activation significantly increased the PI hydrolysis stimulation induced by the Group I agonist DHPG. The potentiation of IP_3 generation with the combined DHPG and LY354740 resulted in a response close to that produced by ACPD alone. Calcium-imaging of the neocortical slices demonstrated that Group II antagonists decreased the fluorescent signal (using Fura-2, correlated with an increase in $[Ca^{2+}]_i$) evoked by ACPD. They also demonstrated in both biochemical assays and calcium

imaging experiments that the addition of a membrane-permeant analogue of cAMP (8-(4-Chlorophenylthio)-cAMP) reversed the stimulatory effects of the Group II. They concluded that Group I mGluR-stimulated PPI hydrolysis in the neocortex was potentiated by concomitant activation of Group II mGluRs and that this might involve a cAMP dependent mechanism.

Another recent study in the neocortex found very similar results. The Group II mGluR agonist had no effect on basal levels IP_3 accumulation, but enhanced the maximal IP_3 response to the Group I mGluR agonist DHPG by almost 100% in both hippocampus and cortex (Mistry et al. 1998). An important component of their study was demonstrating that the potentiation of the IP_3 accumulation was specific to mGluR agonists. Although both the Group I mGluR agonist DHPG and muscarinic acetylcholine receptor (mAChR) agonist carbachol stimulated similar IP_3 accumulations, only the response to the mGluR agonist was enhanced by concomitant activation of Group II mGluRs.

Taken together, these findings generated an interesting hypothesis for the metabotropic pathways underlying the mGluR-mediated ADP in neocortical cells. As most of our early characterization of the properties of the mGluR-mediated ADP in neocortex had relied upon ACPD (an agonist at both Group I and II mGluRs) it seemed reasonable to hypothesize that the response to ACPD may have been a Group II-mediated potentiation of a Group I response. I had demonstrated that activation of Group I mGluRs were responsible for the ADP and that the amplitude of the ADP was dependent in part upon the amount of calcium release from IP_3R-sensitive stores. I had also shown that

Group II mGluR activation alone had no effect on the afterpotential. I now wanted to determine if concurrent Group II mGluR activation could potentiate the physiological response (ADP) mediated by Group I mGluRs. If this were the case, it would not only suggest that an increase in IP_3 levels mediated by the concurrent Group II mGluR activation underlies the increase in ADP amplitude, it would demonstrate a physiological correlate to the biochemical studies reviewed above.

Potentiation of Group I ADPs

A control ADP was evoked using the Group I mGluR agonist DHPG [10μm]. After establishing a consistent response for 10-15 minutes, the Group II mGluR agonist DCG-IV [10μM] was added to the perfusate to combine with DHPG. Because the amplification of the voltage response (ADP) could be the result of the same conductance activated in the setting of increased resistance, both the input resistance and resting membrane potential were monitored before and after the addition of DCG-IV. The amplitude of the ADP was then observed for changes. The Group I-mediated ADP amplitude increased in size within 5-10 minutes of addition of DCG-IV to the bath (Figure 17). As before, data was pooled into 5-minute groups, averaged, and then compared with control records. The amplitude was significantly larger than control ADPs after the addition of the DCG-IV for 2-spike, 5-spike, and 10-spike stimuli ($n = 4$, $p < 0.05$) (not all stimuli were tested in all cells). The mean increase in ADP amplitude for the 5-spike stimulus was 28.6 % (+/- 2.5%) (n=3). There was no observable change in membrane potential after exposure to DCG-IV and the effect of the observed small

increase in input resistance on ADP amplitude was determined by comparing changes in the ratio (before and after addition of DCG-IV) of hyperpolarizing test pulse amplitude to the ADP amplitude. The increase in input resistance was not sufficient to account for the potentiated ADP amplitude after addition of DCG-IV. The order of application of mGluR agents did not influence the results. DCG-IV could be applied prior to or after the introduction of the DHPG and still result in potentiation of Group I response (see previous section on Group II agonist experiments). Figure 18 illustrates an example of the influence of Group II mGluR activation and the spike dependent properties of the ADP. In this cell, the ADP from a 10-spike stimulus in the presence of DHPG was potentiated above spike threshold by the addition of DCG-IV, which resulted in repetitive firing. Although the time course was longer, the potentiation was reversible to either baseline (with DCG-IV only) or by washing out the DCG-IV and returning to the Group I agonist alone (data not shown). Regardless of whether or not this potentiation of the ADP is due to a DCG-IV-mediated increase in DHPG potency or efficacy (see previous citations), this demonstrates a probable physiological manifestation of the biochemical studies. These data suggest that activation of Group II mGluRs can potentiate a response dependent upon the amount of IP_3 generated and subsequent calcium released. In addition, these data confirm the presence of Group II mGluRs on the postsynaptic neocortical neuron.

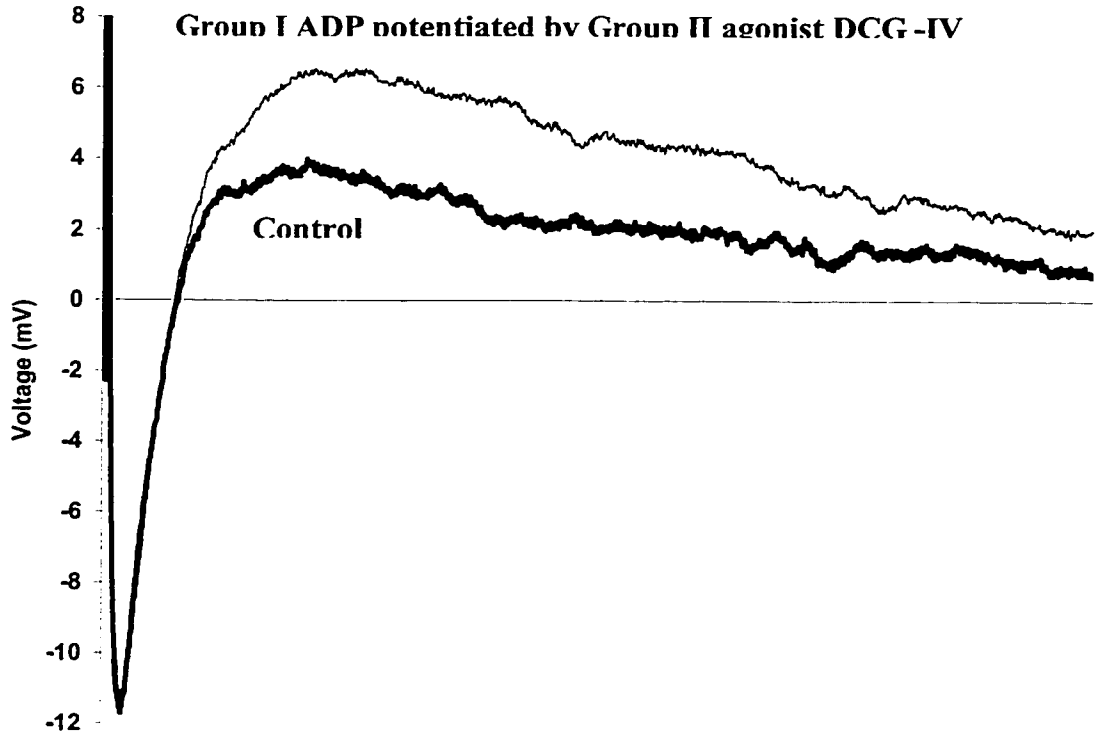

Figure 17. A Group II mGluR agonist potentiates the Group I mGluRmediated ADP. *Bottom Trace (dark line)*: The control ADP was evoked in the presence of the Group I mGluR agonist DHPG [10μM]. *Top trace (light line)*: Group II mGluR agonist DCG-IV [10μM] was then added to the perfusate. This resulted in a potentiation of the ADP. *Both traces*: Data is from one representative cell. Preceding 10-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Traces shown are each an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 4 seconds of sampled time.

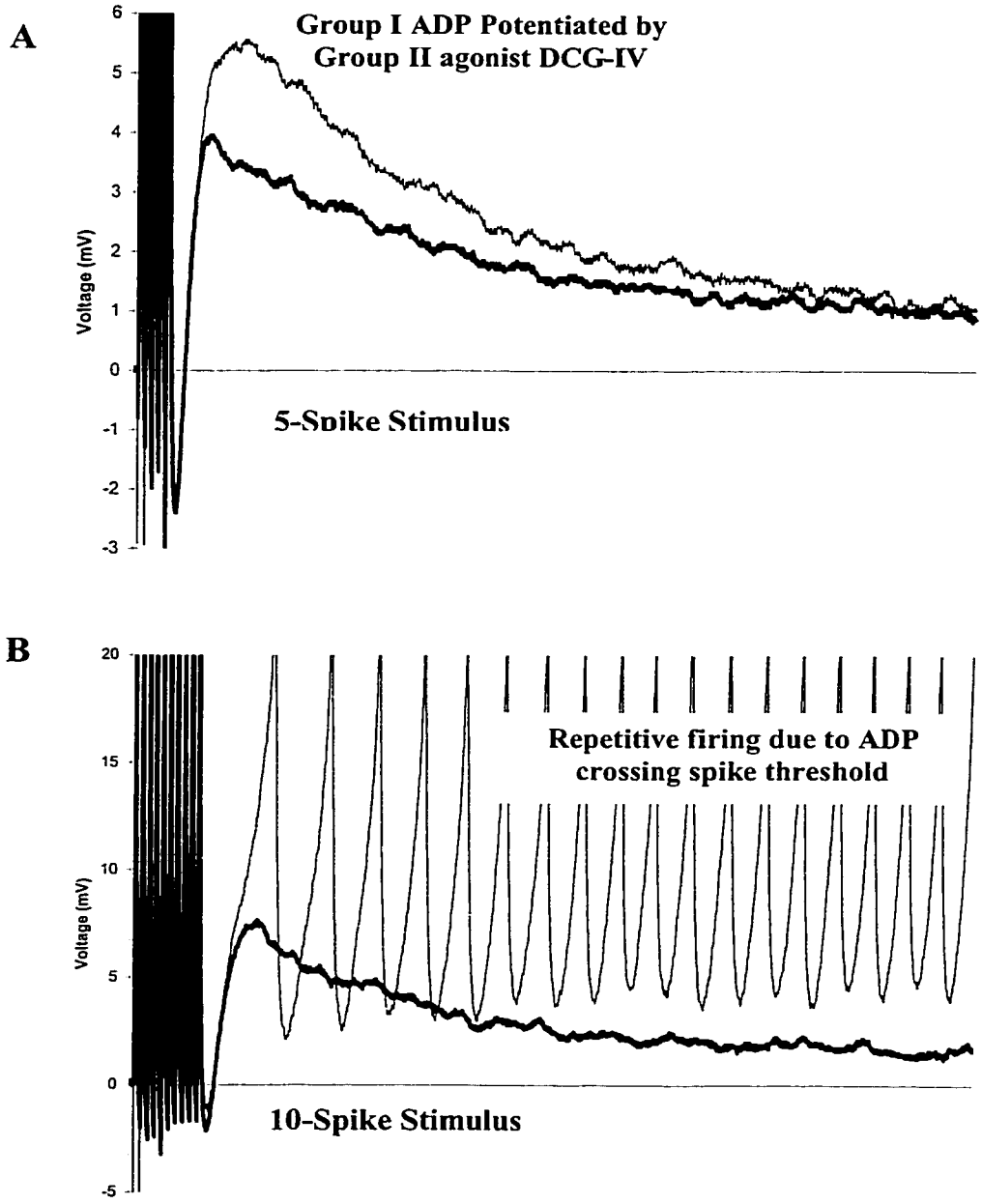

Figure 18. The Group II agonist DCG-IV [10μM] potentiated the Group I mGluR-mediated ADP above spike threshold when the stimulus was increased from 5 to 10 spikes in the same cell. A. *Bottom Trace (dark line)*: Control ADP evoked by 5 spikes in DHPG [10μM]. *Top Trace (light line):* The potentiated ADP evoked by 5 spikes in combination of DHPG [10μM] and DCG-IV [10μM]. **B.** Same Cell. *Bottom Trace (dark line)*: Control ADP evoked by 10 spikes in DHPG [10μM]. *Top Trace (light line):* The potentiated ADP evoked by 10 spikes in combination of DHPG [10μM] and DCG-IV [10μM] crosses spike threshold and results in repetitive firing. Note difference in scale (voltage) between the two figures. In both figures, traces shown are each an average of ~10 records acquired over 5 minutes, except for repetitive firing trace. Axis is equivalent to approximately 4 seconds of sampled time.

Potentiation of mGluR5-mediated ADPs

Experiments had determined that the mGluR-mediated ADP in neocortical neurons was caused by the activation of both of the Group I subtypes (mGluR1 and mGluR5) and dependent upon the release of calcium from both IP_3R- and ryanodine-sensitive intracellular stores. There was now data suggesting that the ADP studied by Greene et al (19994) using ACPD as the mGluR agonist was probably a response mediated by both Group I and Group II mGluRs. To carry the potentiation story further, it would be interesting to know if a specific mGluR receptor subtype interacted with the Group II activation to result in the larger ADP response (presumably via an increase in PPI hydrolysis, IP_3 generation, and calcium release from IP3-sensitive intracellular stores). Because there is not a good selective mGluR1 agonist available, the potentiation experiment was tried with the mGluR5 specific agonist CHPG.

A control ADP was evoked using the mGluR5 agonist CHPG [1mM]. The Group II mGluR agonist was then added to the perfusate and the ADP was observed over time for changes in amplitude. In three cells the mGluR5-mediated ADP increased in amplitude with concurrent exposure to DCG-IV. In one cell the increase was 43%, the other increased by 15%. Another cell also showed potentiation of the mGluR5- mediated ADP, but the potentiation resulted in repetitive firing, indicating that the lower boundary of ADP amplification was at least spike threshold (an amplification of ~59%). Figure 19 illustrates some of the representative findings from these experiments. Again, as seen in the experiments with DHPG and DCG-IV, the potentiating action of the Group II agonist could cause the ADP to cross spike threshold and result in uncontrolled repetitive firing.

Figure 19 also demonstrates how the potentiation of the mGluR5 ADP was so robust that the experimental value (ADP amplitude post DCG-IV exposure) could not be evaluated at the normal resting potential. By hyperpolarizing the membrane potential negative to normal resting potential by ~5mV and keeping the potentiated ADP at a sub-threshold amplitude, one could then measure a potentiated mGluR5-mediated ADP.

These data do not allow comment on the possibility of mGluR1 potentiation, but do suggest that the mechanism responsible for the Group II-mediated potentiation of the Group I response is in part mediated by interaction with the mGluR5 receptor.

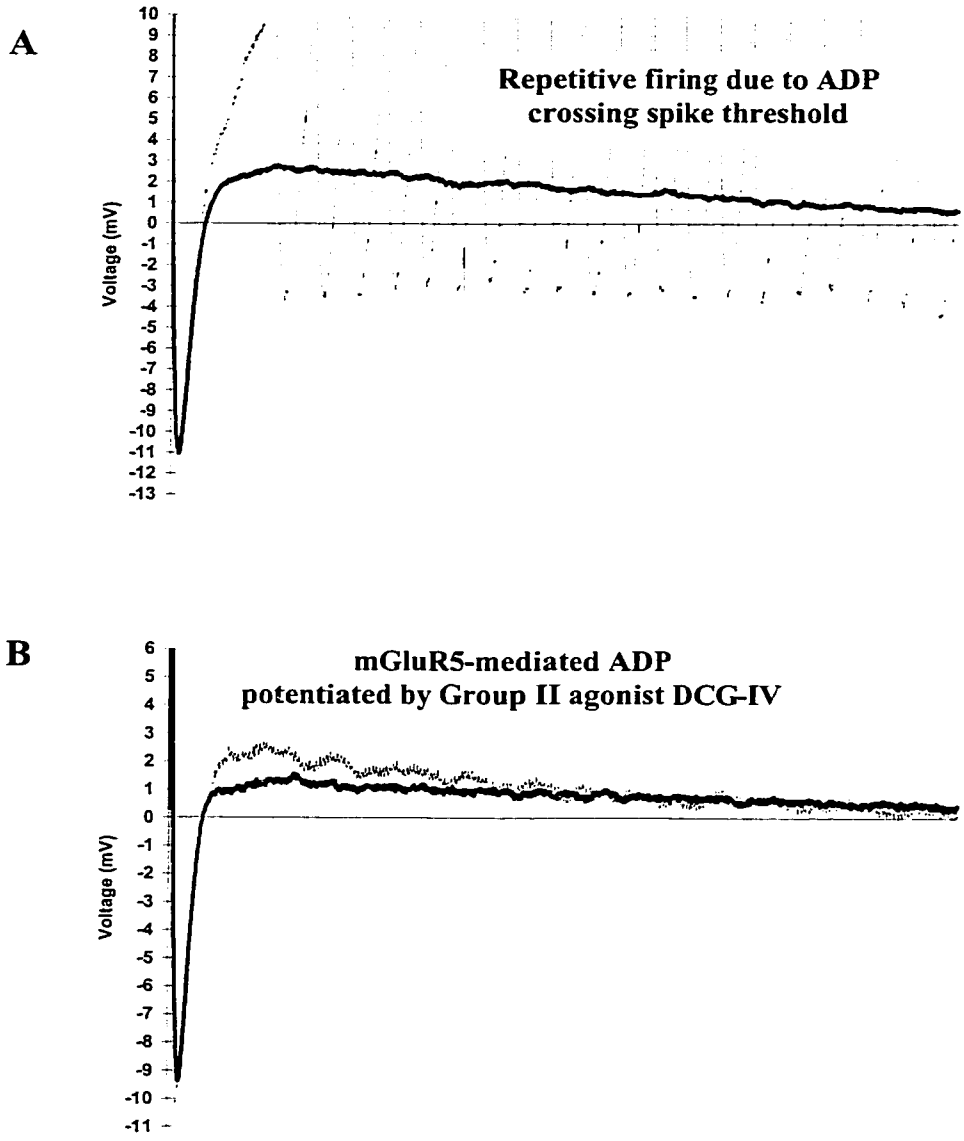

Figure 19. Concurrent activation of Group II mGluRs potentiates the mGluR5-mediated ADP and can in some cases cross spike threshold and result in repetitive firing. A. *Bottom Trace (dark line)*: Control mGluR5-mediated ADP evoked by 5 spikes in CHPG [1mM]. *Top Trace (light line):* The potentiated mGluR5-mediated ADP evoked by 5 spikes in combination of CHPG [1mM] and DCG-IV [10μM] crosses spike threshold and results in repetitive firing. **B.** Same cell held at Vm ≈ –74mV. *Bottom Trace (dark line)*: Control mGluR5-mediated ADP evoked by 5 spikes in CHPG [1mM]. *Top Trace (light line):* Holding the membrane potential hyperpolarized allows the potentiated mGluR5-mediated ADP evoked by 5 spikes in combination of CHPG [1mM] and DCG-IV [10μM] to be observed. Note difference in scale (voltage) between the two figures. In both figures, traces shown are each an average of ~10 records acquired over 5 minutes, except for repetitive firing trace. Axis is equivalent to approximately 4 seconds of sampled time.

ADP Potentiation is Specific to mGluR Interaction

The activation of muscarinic acetylcholine receptors (mAChRs) also stimulates PPI hydrolysis and IP_3 generation in cortex, resulting in calcium release from IP_3R-sensitive stores (Downes 1982). The mAChR-mediated slow ADP previously described in our lab had very similar calcium dependent properties to the mGluR-mediated ADP (Schwindt et al. 1988). However, the dependence of the mAChR-mediated ADP on the calcium released from the IP_3R-sensitive intracellular store had not been demonstrated directly. If this ADP was dependent upon the IP_3R-sensitive stores, could the levels of IP_3 (and release of additional calcium from those stores) be potentiated by concurrent Group II mGluR activation? In two of the biochemical studies previous mentioned of Group II mediated amplification of Group I IP_3 responses, researchers had also looked at the ability of Group II mGluR activation to potentiate the response mediated by mAChRs in hippocampus and cortex (Nicoletti et al. 1993; Mistry et al. 1998). Neither study was able to demonstrate an increase in IP_3 levels. I had demonstrated a physiologic correlate to the biochemical studies of Group II-mediated potentiation of IP_3 levels, and wondered if the physiologic potentiation was specific to interaction of the mGluRs. Would be possible to potentiate the mAChR-mediated ADP with concurrent Group II mGluR activation as well?

First of all, it was necessary to determine if contribution from the IP_3R-sensitive calcium store was essential to the generation of the mAChR-mediated ADP. As in the previously described experiments, low molecular weight (LMW) heparin (5mg/ml) was added to the patch pipette solution and allowed to diffuse into the intracellular matrix

after whole cell access. Atropine was removed from the control perfusate. The control mAChR-mediated ADP was evoked by spike trains in the presence of the mAChR agonist carbachol [10μM] as soon as possible after whole-cell access and stabilization of the cell. The stimulus (10 individual current pulses at ~100 Hz) repeated every 30 seconds. The peak ADP values from five-minute blocks were pooled, averaged, normalized with respect to the initial control ADP, and then compared to cells where no LMW heparin had been introduced. The peak ADP amplitude decreased with time (Figure 20). These results demonstrate that the Ca^{2+} from IP_3R-sensitive intracellular stores contributes to the required elevation of $[Ca^{2+}]_i$ for mAChR-mediated ADP generation. Estimate of the final effect of heparin on the amplitude of the ADP was taken from the asymptotic value of the exponential fit to the data points in Figure 20. In four cells, LMW heparin (5mg/ml) reduced the peak of the mAChR-mediated slow ADP by ~61%.

In the next series of experiments, a control ADP was evoked using 10-50 spikes in the presence of the muscarinic agonist carbachol [10μm]. After establishing a consistent response for 10-15 minutes, the Group II mGluR agonist DCG-IV [10μM] was added to the perfusate to combine with carbachol. The amplitude of the ADP was then observed for changes. The mAChR-mediated ADP amplitude showed no change with the addition of DCG-IV to the bath for the duration of the experiment in four cells. A representative cell is illustrated in Figure 21. In two cells, atropine [5μM] was added at the end of the experiment to block the mAChR-mediated ADP, leaving only DCG-IV remaining. The afterpotential returned to normal control value as in Figure 16 (data not

shown). As before, data was pooled into 5-minute groups, averaged, and then compared with control records. Activation of Group II mGluRs did not influence the mAChR-mediated ADP, suggesting that the potentiation of the mGluR-mediated response is specific to the interaction of mGluRs.

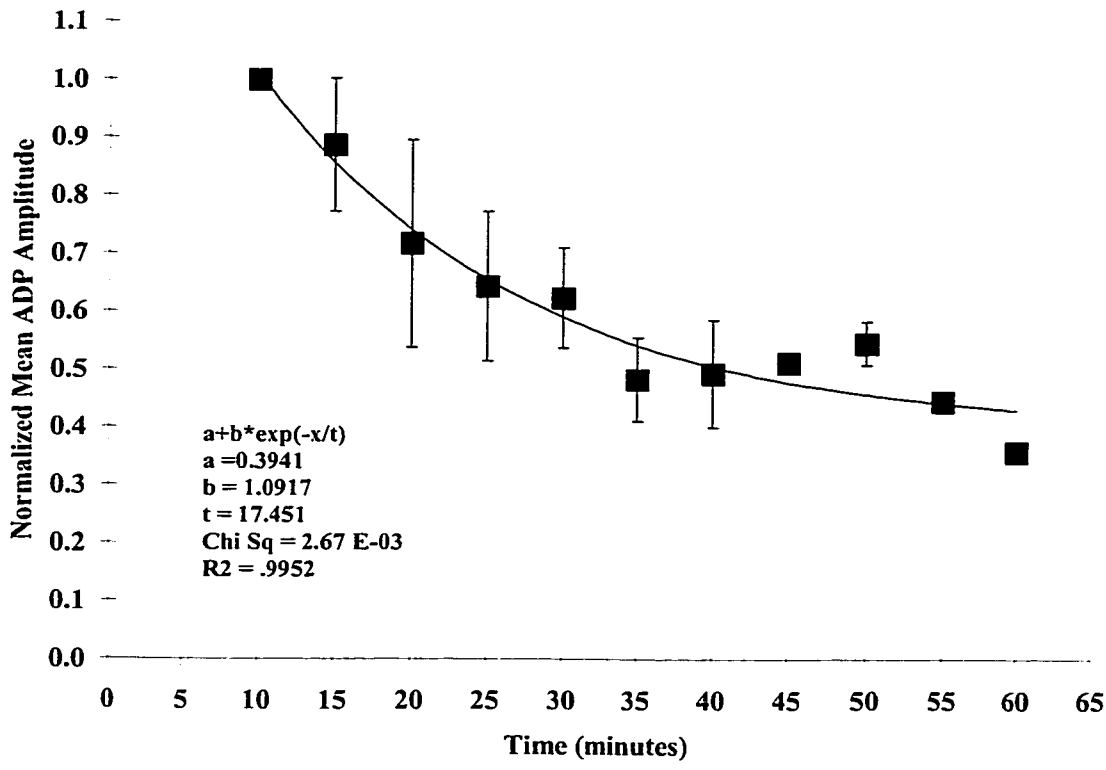

Figure 20. Carbachol-mediated ADP is also dependent on IP_3-sensitive intracellular calcium stores. The mAChR agonist carbachol [10μM] was present continually in the bath. Plotted points (squares) are pooled (5 minute block) averages (+/- standard deviation of the mean) normalized to peak ADP amplitude at onset of experiment (n=4). *Inset*: Fit equation allows estimate of max effect from asymptotic value (decrease of ~61%).

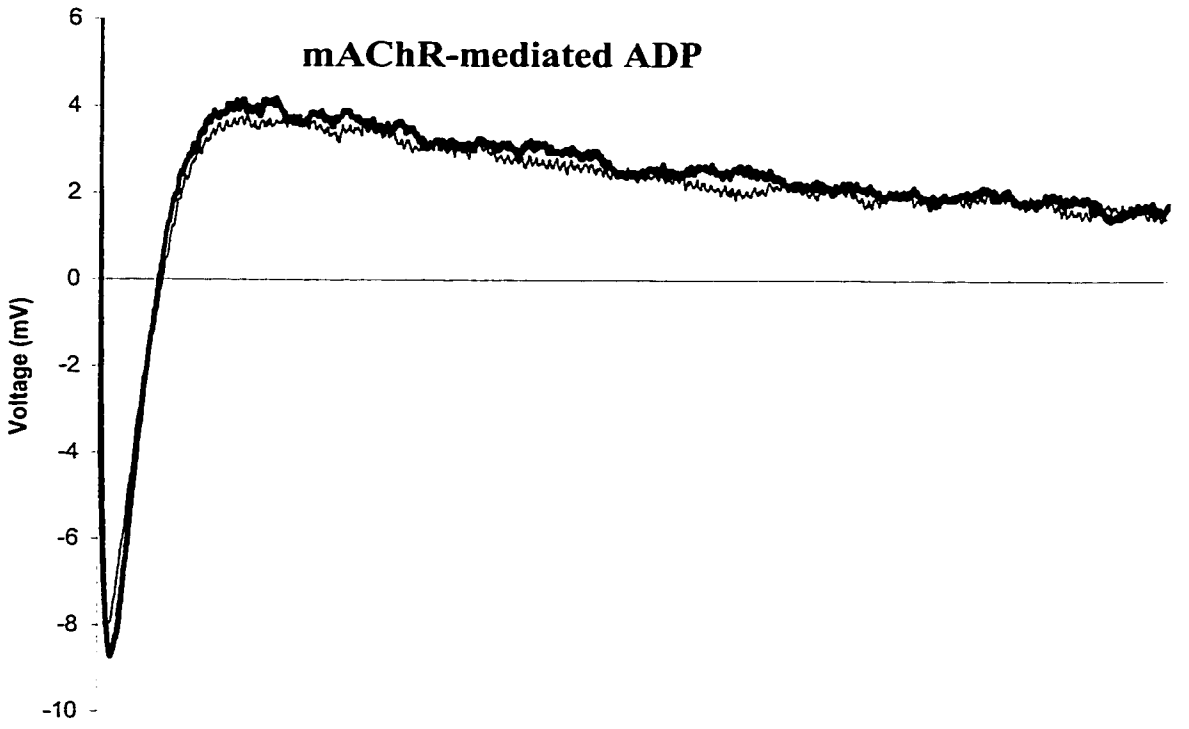

Figure 21. Concurrent Group II mGluR activation does not potentiate the mAChRmediated ADP. *Top Trace (dark line)*: The control ADP was evoked in the presence of the mAChR agonist carbachol [10μM]. *Bottom trace (light line)*: Group II mGluR agonist DCG-IV [10μM] was then added to the perfusate. No potentiation of the ADP was observed for the remainder of the experiment (~45 minutes). Trace shown is at 10 minutes exposure to DCG-IV. Data is from one representative cell. Preceding 50-spike stimulus (generated with individual current pulses at 100 Hz) has been truncated for clarity. Traces shown are each an average of ~10 records acquired over 5 minutes. Axis is equivalent to approximately 3 seconds of sampled time.

Synaptic Stimulation Evokes an mGluR-mediated ADP

Was the mGluR-mediated ADP evoked only in response to pharmacological manipulation of the recorded cell, or could synaptic stimulation, evoking the release of the endogenous mGluR agonist glutamate, induce the mGluR-mediated ADP in the postsynaptic neuron? It is well known that mGluRs can be activated by synaptic activity. There is a wealth of literature on the presence of both pre- and postsynaptic mGluRs at the synaptic junction and their influence on synaptic efficacy (for review see Anwyl 1999). In addition, as reviewed previously in Chapter I, mGluR-mediated nonselective cation currents have been studied using synaptic stimulation (Congar et al. 1997b; Golshani et al. 1998; Heuss et al. 1999; Knopfel et al. 2000). Even the dependence of a synaptically evoked mGluR-mediated nonselective cation current on different intracellular calcium stores had been reported (Partridge and Valenzuela 1999). It seemed likely that the mGluR-mediated ADP in neocortical neurons, suggested by Greene et al (1994) to be due to the activation of a nonselective calcium-dependent cation current, could be evoked with synaptic stimulation. Experiments were then designed to test this hypothesis.

In addition to the ionotropic antagonist kynurenic acid [2mM] and mAChR antagonist atropine [5μM], synaptic stimulation experiments were done with the $GABA_A$ antagonist bicuculline [20μM], $GABA_B$ antagonist 2-hydoxysaclofen [500μM], and the glutamate uptake inhibitor L-trans-2,4-PDC [300μM] in the bath. The GABA antagonists were included due to reports of slow afterpotentials (both inhibitory and excitatory)

mediated by activation of these receptors (Benardo 1994; Cerne and Spain 1997). Dose response experiments with different mGluR agonists in neocortex had suggested that glutamate was a fairly weak agonist when compared to ACPD or quisqualate (Greene et al 1994). Because of this, the glutamate uptake inhibitor was included to maximize the neurotransmitter's exposure to postsynaptic mGluRs.

Synaptic stimulation was evoked by placing an extracellular bipolar electrode on the surface of the slice near the pia. Two different stimulus protocols were tried to elicit an ADP. One method combined both synaptic stimulation (to serve as the "mGluR agonist" source) and injected current into the soma to evoke spikes (to address the spike dependence of the ADP). In two cells this protocol resulted only in a decrease in the amplitude of a hyperpolarized afterpotential (rarely crossing above resting Vm) (data not shown). The other method used high frequency synaptic stimulation (50 individual pulses at 100 Hz), which generated spikes in the postsynaptic cell. A control EPSP (with spikes) was obtained and then the various antagonists listed above were added to isolate the mGluR-mediated response. After the ionotropic antagonists were added to the bath, the evoked EPSPs decreased in size, but were still large enough to cross spike threshold at times throughout the train. This paradigm generated an ADP in one cell. Based on the appearance of the synaptically evoked spikes, I was concerned about the possibility that the block of ionotropic receptors was insufficient, and their stimulation might somehow contribute to the ADP. The dependence of this ADP on mGluR activation could be demonstrated using a specific mGluR antagonist. The most potent and specific Group I mGluR antagonist used to block the ADP in my experiments was the mGluR5 specific

antagonist MPEP. My confidence in the mGluR specificity of this drug was based on electrophysiological recordings in Xenopus oocytes that had demonstrated no significant effect of MPEP at 100 μM on human NMDA, rat AMPA, and human kainate receptor subtypes or at 10 μM on the human NMDA receptor (Gasparini et al. 1999). To confirm mGluR contribution to the ADP, synaptically evoked control ADPs were obtained as described above. Although the number of spikes induced by the EPSP would vary, consistent control EPSP amplitude was obtained. When MPEP [10μM] was added to the bath, the ADP amplitude quickly declined by ~66% within about 5 minutes (Figure 22). The number of spikes evoked by the EPSP decreased in general when compared to the controls, but the difference was not significant enough to account for the change in ADP amplitude. Figure 23 shows an example of both a control ADP and an ADP after the addition of MPEP. Both of these records had identical stimuli of synaptically evoked EPSPs with 20 evoked spikes. This demonstrates that the decline in ADP amplitude after the addition of the mGluR5 antagonist was not due to a decreased number of spikes. The amplitude of the synaptically evoked ADP partially recovered when MPEP was washed out (data not shown). This finding provides the first evidence that an mGluR-mediated ADP in neocortical neurons can be evoked with synaptic stimulation. In addition, the activation of the Group I subtype mGluR5 contributes to the synaptically evoked ADP.

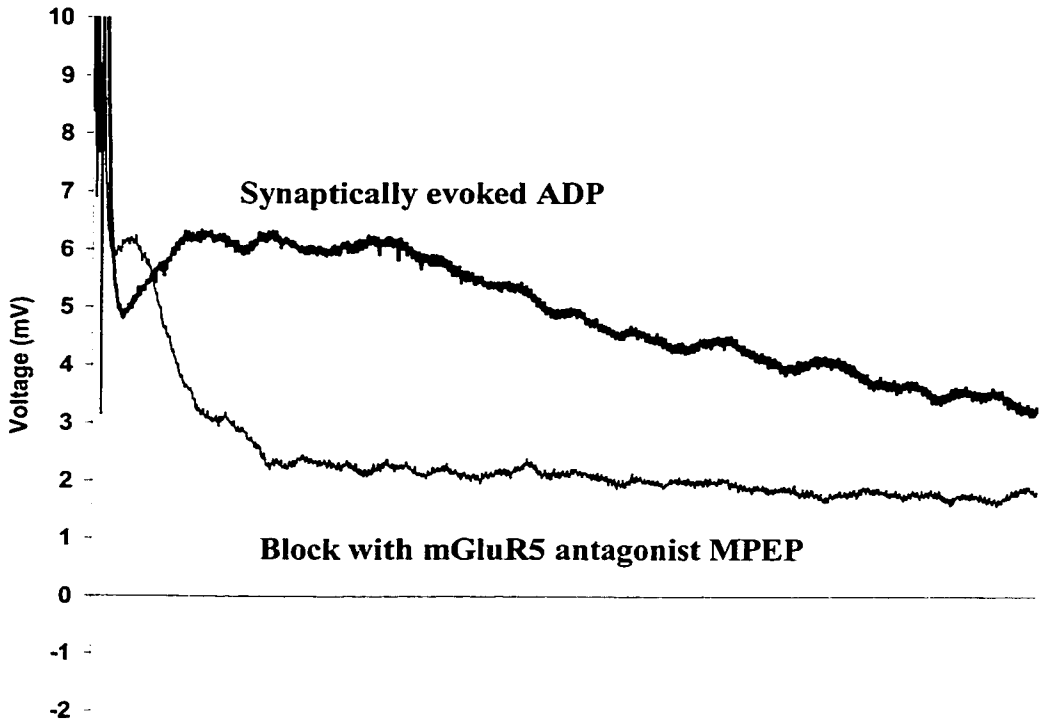

Figure 22 The ADP evoked with high intensity synaptic stimulation is mediated in part by mGluR5 activation. Top trace (dark line): Control ADP using high frequency synaptic stimulation (50 individual stimuli at 100Hz). Kynurenic acid [2mM], atropine [5μM], bicuculline [20μM], 2-hydoxysaclofen [500μM], L-trans-2,4-PDC [300μM] are present in the bath. No mGluR agonist is present. Bottom Trace (light line): ADP amplitude declined to ~66% of control within 5 minutes of adding MPEP [10μM] to the bath. Data shown is from one cell. Only the afterpotential is shown for clarity. Traces shown are each an average of ~5 records acquired over 5 minutes. Axis is equivalent to approximately 3 seconds of sampled time.

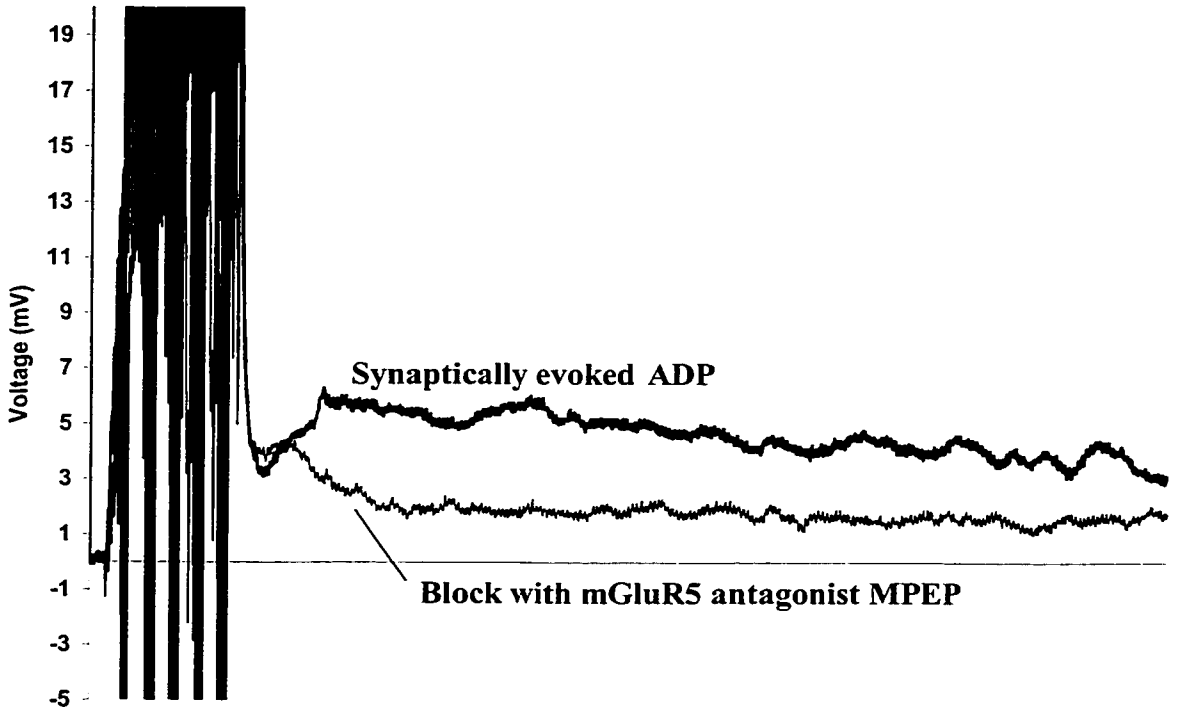

Figure 23 Change in amplitude of synaptically evoked ADP after addition of mGluR5 antagonist was not due to reduced number of evoked spikes *Top Trace (dark line):* Control ADP using high frequency synaptic stimulation (50 individual stimuli at 100Hz). There are 20 spikes evoked by the EPSP. Kynurenic acid [2mM], atropine [5μM], bicuculline [20μM], 2-hydoxysaclofen [500μM], L-trans-2,4-PDC [300μM] are present in the bath. No mGluR agonist is present. Bottom Trace (light line): Using the same stimulus, the ADP amplitude declined after addition of MPEP [10μM] to the bath. There are also 20 spikes evoked by this EPSP. Data shown is from one cell. Representative traces from same cell as in Figure 22. Axis is equivalent to approximately 4 seconds of sampled time.

CHAPTER V: Discussion

The objective of this research was to examine the relationship between mGluR associated biochemical events described in the literature and the mGluR-mediated physiologic response (ADP) observed in layer V cortical neurons. This dissertation describes research that meets this objective, elucidates some of the metabolic pathways activated by mGluR stimulation and the specific mGluR receptor subtypes involved, and provides insight into possible mechanisms contributing to the mGluR-mediated ADP in neocortical neurons.

My major findings can be summarized as follows. The spike-dependent mGluR-mediated ADP can be evoked with synaptic stimulation, relies upon the activation of both mGluR1 and mGluR5 subtypes of Group I mGluRs, requires G-protein activation, and is dependent upon calcium release from both IP_3R-sensitive and ryanodine-sensitive intracellular stores. Activation of Group II mGluRs alone has no measurable effect on the electrophysiologic parameters of neocortical neurons, but does specifically potentiate the Group I-mediated ADP. The following sections will discuss the significance of these findings in more detail and, based on these conclusions and reports from the literature, introduce a plausible hypothesis for the mechanism underlying the mGluR-mediated ADPs in neocortical cells.

The mGluRs Mediating the ADP in Neocortical Neurons

The use of specific pharmacological mGluR agonists and antagonists indicated that the mGluR-mediated ADP in neocortical neurons is due to the activation of both of the Group I mGluR subtypes (mGluR1 and mGluR5). The pharmacological tools chosen for these experiments have been well studied both in terms of their specificity for mGluRs and their mGluR subtype specificity (Anwyl 1999). Considering that the expression of the Group I mGluR subtype mGluR5 is high in the neocortex (Abe et al. 1992; Casabona et al. 1997), it is not surprising that activation of mGluR5 contributed to the generation of the ADP. However, it was interesting to find that S4-CPG, an antagonist that targets the mGluR1 subtype, was able to diminish the amplitude of the ADP since mGluR1 receptors are expressed at much lower levels in the cortex (Martin et al. 1992). My result suggests there is a sufficient level of mGluR1 expression in the neocortex of the rats used in this study (aged 14-24 days) that activation of these receptors also can contribute to the ADP response.

In the population of cells studied it was found that application of supposedly specific antagonists against either mGluR1 or mGluR5 reduced the ADP evoked in the presence of DHPG by more than 50%. This may have resulted from the chance occurrence that one subtype or the other predominated in the small population of specific cells that were tested with each antagonist or indicate that the antagonists are less specific than reported at the doses used in these experimental conditions.

Does the stimulation of these Group I mGluRs activate the conductance underlying the ADP? Previous study of the neocortical mGluR-mediated ADP suggested

that both the activation of a calcium dependent nonselective cation current and the suppression of a calcium dependent resting potassium conductance were responsible (Greene et al 1994). Although the present research did not specifically study the ionic mechanisms underlying the ADP, identification of the mGluR subtypes involved are consistent with the literature concerning Group I mGluR-mediated (Guerineau et al. 1995; Congar et al. 1997b; Chu and Hablitz 2000) and specific mGluR1- (Batchelor et al. 1994) and mGluR5- (Partridge and Valenzuela 1999) mediated activation of nonselective cation currents.

As mentioned in Chapter I, there are reports of changes in relative expression of mGluR subtypes with developmental age. In the developing rat brain, the level of mGluR1 expression gradually increases with age (Shigemoto et al. 1992; Catania et al. 1994), but not notably in the cortex (Casabona et al. 1997) – suggesting that levels begin relatively low and remain that way. Detailed studies of the rat neocortex have shown expression of mGluR5 exclusively in layer V at postnatal days 3 and 7, and then throughout the cortex by day 10 (Bevilacqua et al. 1995), but the overall expression level of mGluR5 in cerebral cortex is then reported to decline, based on comparison to mGluR5 expression in adult rats. (Catania et al. 1994; Romano et al. 1996; Casabona et al. 1997). Therefore, although the expression of mGluR1 in the cortex is low relative to mGluR5, the relative contribution of each of the subtypes to the ADP response may be expected to change with development.

A significant adjunct to our understanding of the types of mGluRs mediating the ADP was the finding that Group II mGluR activation potentiated the Group I response.

Expression of mGluR2 (Tanabe et al. 1992; Neki et al. 1996) and mGluR3 mRNA (Tanabe et al. 1992) is widespread in most neuronal cells of the cerebral cortex. The work from Neki et al (1996) suggested that, in addition to presynaptic expression of mGluR2, there might be postsynaptic expression as well. The data presented here provides physiological evidence for the postsynaptic expression of Group II mGluRs in neocortical neurons. Obviously, if expression of the Group I mGluR subtypes changes with development, it is important to appreciate the relative capacity for each Group I mGluR subtypes to be potentiated by concurrent Group II mGluR activation. My demonstration of the potentiation of the mGluR5-mediated response does not exclude the possibility of mGluR1 potentiation as well. Further discussion of the potentiation of the Group I response will continue in a later section.

Synaptic Stimulation of mGluR-mediated ADP

The experiments studying the mGluR-mediated ADP in neocortical neurons to date had relied upon specific pharmacological stimulation of the mGluRs via the bath perfusion of agonists. Normally, the endogenous neurotransmitter for these receptors is glutamate. Glutamate was tested as a bath agonist (Greene et al 1994), but found to be considerably less potent than ACPD and was not used for further study of the ADP. Any potential physiologic consequences of the ADP demanded a demonstration that synaptic stimulation could produce an ADP similar to that observed when using bath application of agonists. The results presented here demonstrate that presumably glutamatergic

synaptic stimulation can evoke an mGluR-mediated, spike-dependent ADP in neocortical neurons.

As reviewed in Chapter I, synaptic stimulation has been shown to evoke mGluR-mediated responses in various areas of the brain. In most cases, high frequency stimulation (~1 second long at 100Hz) was required to elicit the response. Nonselective cation currents, depolarizing EPSPs, or slow ADPs attributed to the activation of Group I mGluR-mediated in thalamic and hippocampal CA3 neurons (Congar et al. 1997b; Golshani et al. 1998; Heuss et al. 1999), mGluR1-mediated in cerebellar and hippocampal CA3 neurons (Batchelor et al. 1994; Heuss et al. 1999; Knopfel et al. 2000), and mGluR5-mediated in hippocampal CA1 neurons (Partridge and Valenzuela 1999). When investigating the appropriate synaptic stimulus to produce an mGluR-mediated ADP in the neocortical neurons, I first evoked EPSPs that were subthreshold for spike initiation before the application of the ionotropic antagonists, and the EPSPs were virtually eliminated by the ionotropic antagonists. Thus, the concurrent postsynaptic spikes were evoked by intracellular current injection. The timing of the postsynaptic spikes was varied in relation to the synaptic stimulus. This stimulus resulted in a marked reduction in the amplitude of the AHP in two cells. It is unknown if this change in the AHP was a reflection of activation of a small, inward, ADP current or some other mechanism. There are two possible reasons why this combined stimulus did not evoke an ADP. The synaptic stimulus may have been too brief to generate the necessary glutamate concentration for adequate mGluR activation or, the time course of the action potentials and associated VGCC calcium influx may need to be precisely correlated

spatiotemporally with the mGluR-mediated calcium release from intracellular stores to maximally activate the responsible ADP conductance. The need for a spatiotemporal correlation has been shown in other preparations. When a back propagating action potential was paired with synaptic activation of mGluRs in CA1 hippocampal neurons the resulting calcium entry through VGCC resulted in an amplified calcium release from IP_3R sensitive stores nearest the site of the synaptic input (Nakamura et al. 1999).

The synaptic stimulus protocol that did evoke an ADP in this study (50 stimuli at 100 Hz) was strong enough to result in postsynaptic spike generation. After the ionotropic antagonists were added to the bath, the evoked EPSPs decreased in size, but were still large enough to cross spike threshold at times throughout the train. After the addition of the mGluR5 antagonist MPEP, there were fewer spikes evoked, but the underlying EPSP was not discernibly smaller. This suggests that mGluR5 activation may have contributed to the postsynaptic depolarization and subsequent spike generation. The reduced number of spikes evoked (reduced VGCC calcium entry) after the mGluR5 antagonist did not seem to play a significant role in the reduction in ADP amplitude. This observation could be explained by the reduced influence of continued calcium influx from a spike train after ~20 spikes on ADP amplitude (See Figure 2C). The residual EPSPs may have resulted from incomplete block of ionotropic receptors, or from nonspecific cation currents evoked by mGluR stimulation, or from both causes. Nevertheless, the combination of adequate synaptic input and postsynaptic spikes evoked an ADP. The large reduction of this synaptically evoked ADP by the specific mGluR5 antagonist MPEP confirms that it depended in part on the synaptic activation of mGluR5.

This result is consistent with the bath mGluR agonist experiments in that the ADP generation required spikes as well as mGluR stimulation. The need for strong, high frequency synaptic stimulation to evoke the ADP under conditions where glutamate uptake was impeded is supportive of a pathophysiologic role of Group I mGluR-mediated responses in excitotoxic conditions associated with an abnormally elevated extracellular glutamate concentration (Choi et al. 1987; Strasser et al. 1998)

G-Protein Dependence and Second Messengers

As reviewed in Chapter I, there are reports of G-protein independent mGluR-mediated responses (Guerineau et al. 1995; Heuss et al. 1999), and various Group I mGluR-mediated responses have been reported due to generation of 1,2-diacylglycerol (DAG) and activation of protein kinase C (PKC). There is also growing body of literature implicating tyrosine kinase (Abdul et al. 1996b; Umemori et al. 1997; Heuss et al. 1999) and mitogen-activated protein kinase (MAPK) (Kurino et al. 1995; Peavy and Conn 1998; Ferraguti et al. 1999). While the results of my experiments do not exclude the possibility that other mechanisms may play a role, they indicate an absolute dependence the ADP on the participation of a G-protein and the generation of IP_3.

The Role of Calcium in the mGluR-mediated ADP

Greene et al (1994) found that the ADP is dependent on stimulation of mGluRs, calcium influx through VGCCs, and a rise of intracellular calcium. It is assumed that the primary role for the intracellular calcium elevation is to activate the calcium-dependent

cation conductance and to suppress the resting potassium conductance inferred by Greene et al (1994) to underlie the ADP. In rat hippocampus, combined voltage-clamp recordings and microfluorometric measurements of $[Ca^{2+}]_i$ using the Ca^{2+} indicator fura-2 revealed that the amplitude of the current underlying an mGluR-mediated ADP was correlated with the amplitude of depolarization-induced Ca^{2+} influx (Caeser et al. 1993). In another study, the addition of calcium from either an exogenous source (electrode solution) or stimulation of release of calcium from intracellular stores resulted in a potentiation of a nonselective cation channel-dependent depolarization in rat hippocampus (Partridge and Valenzuela 1999).

Since spike trains do not evoke ADPs in these cells in the absence of mGluR stimulation, a mechanism other than calcium influx through VGCC is necessary for the elevation of intracellular calcium to a level sufficient to cause the ADP. The present experiments indicate a dependence of the mGluR-mediated ADP on release of calcium from both the IP_3R-sensitive and ryanodine sensitive intracellular stores. Blocking release of calcium from either intracellular store significantly reduced the amplitude of the ADP. The data also suggests that blocking release from both stores concurrently ultimately results in the elimination of the ADP, but the time course of ADP decline was much slower than when release from only one of the stores was blocked. These results shed more light on the mechanisms underlying the generation of the mGluR-mediated ADP, but the of precise role of each type of intracellular calcium store in ADP generation is still unclear and will require additional future investigation.

In the meantime, the following hypothesis is proposed to reconcile the observations from the present experiments, the known properties of the ADP, and current reports from the literature. An explanation is required for both "the ADP" and its temporal properties. In the layer V cells an ADP appears after a single spike in the presence of Group I mGluR agonists, but the ADP amplitude increases to a maximum as the number of spikes in a train (evoked at 100 Hz) are increased to 5-10, and then the amplitude declines (but more slowly – see Figure 2) as more spikes are added to the train. Figure 24 is a cartoon that illustrates the main points of this hypothesis which may be summarized as follows: (1) Group I mGluR stimulation raises intracellular calcium concentration above its resting level but not to a level to reach "threshold" for the ADP conductances nor to a level that significantly activates calcium induced calcium release (CICR) from ryanodine sensitive stores. (2) Calcium influx through VGCC is critical for ADP generation. Its role is twofold. It increases the sensitivity of the IP_3Rs to IP_3 so that more calcium is released from these stores and, together with the raised calcium from IP_3-sensitive stores, triggers CICR. (3) This positive feedback causes intracellular calcium to cross the "threshold" of ADP conductance activation (following a single spike for the layer V neurons). (4) Subsequent spikes in a train enhance this positive feedback by providing additional influx of calcium, causing the ADP amplitude to grow until the process begins to decay by one or more of several possible mechanisms. (5) A prime mechanism for this is the well known bell-shaped relation between IP_3R channel activity and calcium concentration at a given IP_3 level. Increasing calcium concentration causes decreased binding of IP_3 to its receptor, less calcium is released from the IP_3R-sensitive

stores, and there is insufficient calcium to enhance the ADP. Intracellular calcium and ADP amplitude thus decline with spike number beyond the optimum at a given stimulus rate. The slower decline in ADP amplitude with increasing number of spikes is due to continued VGCC calcium influx and a shift in stimulation to a population of lower affinity IP_3R subtypes that allow the maintenance of calcium release during periods of prolonged stimulation. It is possible that the ADP conductances themselves also are inactivated by excess calcium, and this also contributes to the ultimate decline of ADP amplitude with increasing number of spikes at a given rate. The tenets of this hypothesis and their relation to the experimental observations are discussed in more detail below.

Group I mGluR activation elevates intracellular calcium concentration via IP_3R-sensitive release.

The ADP requires Group I mGluR activation, which is known to cause PI hydrolysis and generate levels of intracellular IP_3 to rise. The IP_3 serves as an agonist for calcium release from IP_3R-sensitive intracellular calcium stores. I have shown that blockade of IP_3Rs with heparin reduces the ADP amplitude, indicating a dependence of the ADP on increased IP_3 production. The elevation of baseline intracellular calcium concentration with Group I mGluR activation is well documented by calcium imaging studies (some examples include Llano et al. 1991; Jaffe and Brown 1994; Maiese et al. 1999).

Calcium influx via VGCCs (during spikes) boosts calcium release from the IP_3R-sensitive intracellular store.

IP_3Rs have two separate binding sites for Ca^{2+} and IP_3, and are regulated allosterically by these two ligands with binding of one ligand facilitating additional binding of the other (Berridge 1998; Hirose et al. 1998). Calcium cannot open the channel in absence of IP_3, but in the presence of IP_3 it can increase the activity of the IP_3 receptor (Finch et al. 1991). Within physiologic ranges of intracellular calcium, the IP_3R channel activity responds with both positive and negative feedback, describing a "bell-shaped" response curve to changes in intracellular calcium concentrations (Bezprozvanny et al. 1991). Calcium is the only known physiological inhibitor of IP_3 receptors. Recent data describing the discovery of both high and low affinity IP_3Rs have modified the original interpretation, providing a model that continues to allow the cell to minimize the rise of intracellular Ca in the presence of low levels of IP_3 (the "bell shaped" curve), but also provides a means of maintaining higher intracellular Ca^{2+} during periods of prolonged stimulation (Kaftan et al. 1997; Moraru et al. 1999). It has also been demonstrated in the neocortex that, after IP_3Rs have been sensitized beforehand (by activation of mAChRs in this case), calcium entry via VGCCs can serve as the actual messenger that opens the IP_3R-sensitive channel (described as IP_3R-assisted CICR) (Yamamoto et al. 2000). VGCC calcium influx has also been shown to stimulate IP_3R-sensitive calcium release in CA1 pyramidal neurons (Nakamura et al. 1999).

Additional spike-evoked calcium influx will, in combination with the elevated calcium already present, trigger CICR from ryanodine-sensitive intracellular stores.

It is now possible to describe CICR from both ryanodine-sensitive and IP_3R-sensitive intracellular stores, so the observation of the ADP amplitude increasing with number of spikes does not discriminate which intracellular calcium store is being triggered by the VGCC calcium influx. The CICR is probably stimulated primarily by VGCC calcium entry, but it may also be triggered in part by the calcium release from IP_3R-sensitive stores. However, the calcium from IP_3R-sensitive release cannot trigger significant CICR since an mGluR-mediated ADP does not occur without spikes. The data presented here found that the block of CICR from ryanodine-sensitive stores decreases ADP amplitude considerably, suggesting a prominent role for CICR. Supporting the hypothesis that the ryanodine-sensitive store is triggered by VGCC calcium entry is the IP_3R's property of negative feedback at elevated levels of intracellular calcium. Cytoplasmic calcium concentrations above [0.3 μM], a level well within the range of intracellular calcium levels that are found after cellular stimulation, decrease binding of IP_3 to the receptor. Calcium also inhibits the ryanodine receptor but only at millimolar concentrations (Ehrlich et al. 1994). Because of the functional capacity of the ryanodine receptor in a broader range of calcium concentration, it is more likely to participate as the additional VGCC-mediated calcium entry causes increasingly elevated $[Ca^{2+}]i$. As mentioned above, VGCC calcium entry has been shown to be sufficient for regenerative, all or none CICR from ryanodine sensitive stores in rat sensory neurons (Usachev and Thayer 1997). In addition, VGCC stimulus of CICR is supported by recent reports that

found that Ca^{2+} release from ryanodine-sensitive stores contributed to Ca^{2+} signals triggered by action potentials in hippocampal CA1 neurons (Sandler and Barbara 1999).

Once above the threshold for activation of the calcium-dependent conductance(s), the increasing number of spikes cause continued elevation in intracellular calcium, and the stepwise VGCC calcium entry correspondingly triggers CICR from ryanodine-sensitive intracellular calcium stores (Usachev and Thayer 1997). This additional source of calcium can, as cited earlier, further amplify the ADP conductance (Caeser et al. 1993; Partridge and Valenzuela 1999). The proposed mechanism (CICR) allows a dynamic change in intracellular calcium dependent on the spike train.

Eventually, the contribution of CICR (stimulated by both VGCC influx and IP_3R-sensitive release) to the activation of the conductance underlying the mGluR-mediated ADP would peak. Supporting this idea is the IP_3R's property of negative feedback at elevated levels of intracellular calcium. Thus, as intracellular calcium concentration rises this negative feedback would limit it's maximal rise and thus maximal ADP amplitude at a given stimulus rate. The inhibitory influence of high calcium concentrations on IP_3R - or ultimately even ryanodine receptor – activity would inhibit release from these stores to cause a decline in ADP amplitude in the face of continued calcium influx through VGCC (increased number of spikes). Although the ADP amplitude gradually declines with increasing number of spikes and loss of contributory calcium from intracellular stores, as long as there is continued mGluR stimulation and spike stimulus, the ADP is not completely eliminated.

Experimental support

In the present experiments, blockade of CICR with ryanodine reduced but did not eliminate the ADP. Assuming the dose of ryanodine was saturating, I would conclude that VGCC calcium influx and IP_3R-sensitive calcium release are still capable of crossing the ADP threshold and generating a small ADP, small because the normal "amplification" of the ADP reliant on the CICR is unavailable.

Blockade of IP_3Rs with heparin also resulted in a significant decline in ADP amplitude, though again not in a complete block. The foundation of the hypothesis put forth above rests on calcium release from IP_3 sensitive stores, and according to the hypothesis, blockade of IP_3Rs by a saturating dose of heparin should eliminate the ADP. The VGCC calcium entry and CICR are not sufficient to activate the ADP conductance without the initial IP_3R-mediated elevation in baseline calcium concentration. ADPs are not observed with only spike trains. Assuming that the heparin dose employed was saturating, a possible explanation for the failure of heparin to eliminate the ADP is the stimulation of CICR from ryanodine-sensitive stores by heparin. Although heparin is a commonly used antagonist of the IP_3R, it also induces intracellular calcium release from ryanodine-sensitive stores (Ritov et al. 1985), and it has been shown to directly activate ryanodine receptors in single channel studies (Bezprozvanny et al. 1993; Ehrlich et al. 1994). Acting as a polyanion, heparin changes the charge around the ryanodine receptor which attracts calcium ions and activates CICR. A recent study demonstrated inhibition of the ryanodine receptor by the heparin antagonist protamine (Koulen and Ehrlich 2000).

The block could be reversed by the addition of heparin. The authors suggest that protamine acts on the ryanodine receptor in a similar but opposite manner from heparin.

If heparin stimulated calcium release from ryanodine-sensitive intracellular stores in the present experiments, then this pharmacologically evoked CICR from ryanodine-sensitive stores may have summed with the calcium influx from VGCC (which influx may have triggered even more CICR) to raise calcium above the ADP conductance "threshold". The heparin activation of the ryanodine receptor is also calcium dependent, becoming more effective with increases in intracellular calcium concentration (Bezprozvanny et al. 1993). This property would support the ability of the VGCC calcium entry to enhance the heparin-mediated CICR sufficiently to cross the threshold for ADP generation.

The alternative explanation for the results discussed above, less than saturating doses of heparin or ryanodine, seems unlikely because the doses chosen were considerably higher than most others cited in similar studies and the experiment using combined heparin and ryanodine to block intracellular calcium release from both stores resulted in a significant decrease in the amplitude of the ADP. Extrapolation of the exponential fit of the data suggested that the ADP would have been eventually eliminated, further suggesting that the effective doses of both blockers were saturating. The time course of the block of the ADP was notably longer than in individual experiments with the respective drugs, however. The activation of ryanodine receptors by heparin discussed above also provides an explanation for this observation. The prolonged time course of the block could result from the competition between heparin

and ryanodine on the population of ryanodine receptors. Heparin activation of ryanodine–sensitive stores would result in a slower decline of intracellular calcium than when either drug was present alone to specifically block its respective store. Ryanodine is quite specific and has not been reported to interact with the IP_3R (Ehrlich et al. 1994).

Future experiments required to test the "roles of calcium" hypothesis

Both the inability of a presumably saturating dose of heparin to eliminate the ADP (which the hypothesis demands) and the slow decay of ADP elimination in the presence of presumably saturating doses of heparin and ryanodine were attributed to the possibility that there is enough heparin-stimulated CICR (when IP_3R-mediated calcium contribution is blocked) to sum with VGCC calcium entry to cross threshold and generate a small ADP. Under these conditions, there is no role of the mGluR agonist, and an ADP should be able to be stimulated without its presence. As none of the cells from the current experiments were tested with heparin alone (the mGluR agonist was present from the beginning of the experiment to establish the control ADP), this is a critical experiment that needs to be performed in the future. If no ADP is generated using a spike train stimulus with a saturating dose of heparin (5 mg/ml) present intracellularly, then this would indicate that the results observed in the present study are most easily explained by an incomplete block of IP_3Rs, and an alternative explanation for the present experimental results must be offered.

A major difficulty to more detailed or conclusive interpretations of the present experimental results is that changes of calcium concentration in relation to drug

application and spike activity rests on speculation – expectations from reports in the literature – rather than observation. Thus, it would be desirable in future experiments to combine the whole-cell patch techniques used here with calcium imaging. The baseline $[Ca^{2+}]i$, subsequent changes in $[Ca^{2+}]i$ after application of mGluR agonist in the perfusate, and the contribution of spike evoked calcium to the $[Ca^{2+}]i$, could be determined. Introducing specific pharmacologic agents through the patch electrode into the cell, one could investigate the contribution of VGCC triggered CICR by using dantrolene or ruthenium red, or alternatively one could test if VGCC calcium entry caused CICR that was blocked by heparin. If carefully done, experiments using single action potentials may determine which intracellular calcium store contribution is critical for reaching ADP threshold.

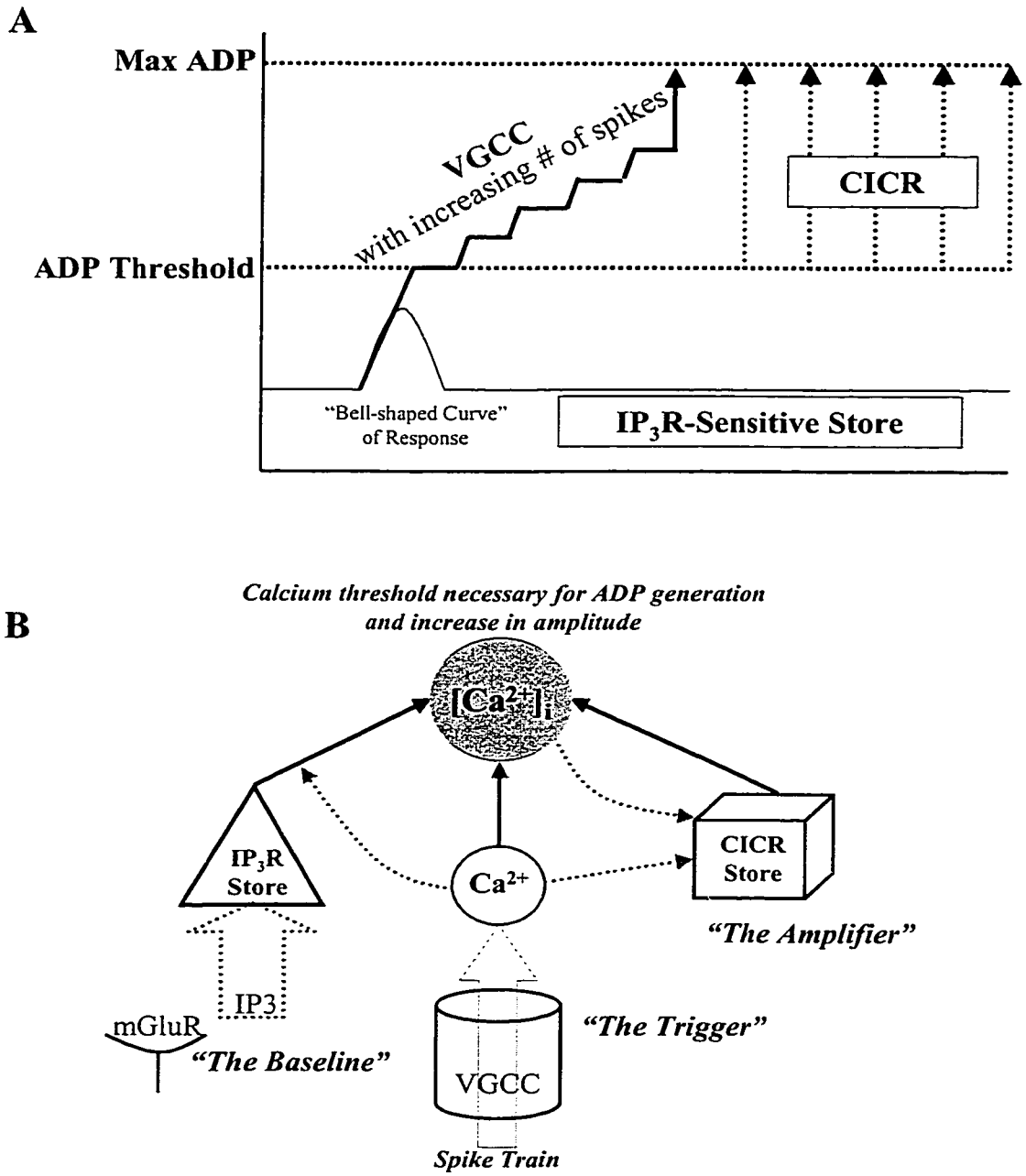

Figure 24. Cartoon illustrating the proposed hypothesis regarding the role of calcium influx and intracellular stores in the generation of an mGluR-mediated ADP. A) Graph depicting changes in calcium level and contribution from each source in the generation of the spike-dependent mGluR-mediated ADP. B) Diagram of the different calcium sources (*solid lines*), the stimuli for that source's contribution (*shown with dotted gray lines)*, and their proposed interaction.

Potentiation of the Group I mGluR-mediated ADP

The data presented in this dissertation demonstrates that stimulation of Group II mGluRs using the specific agonist DCG-IV did not cause an ADP in neocortical neurons, but Group II stimulation significantly potentiates the amplitude of the Group I (DHPG) mGluR-mediated ADP. In addition, it was determined that at least a portion of this potentiation is mediated through the specific Group I subtype, mGluR5. Prior to this, although Group I and II mGluRs are co-expressed in cerebral cortex, only biochemical studies had suggested expression within the same cellular compartment. The results of this research provide physiological evidence that confirms the presence of both Group I and II mGluRs in the postsynaptic neocortical neuron and elucidate the function of the Group II mGluRs.

These results provide the first physiologic correlate in neocortical neurons to biochemical events described in multiple studies on the concurrent activation of Group I/II mGluRs. As reviewed in Chapter IV, the effect of concurrent Group I/II mGluR activation has been primarily published in biochemical studies, looking at increases in PI hydrolysis (Nicoletti et al. 1993; Genazzani et al. 1994; Schoepp et al. 1996; Schaffhauser et al. 1997; Mistry et al. 1998). This result of my research demonstrates a physiological consequence to the reported potentiation of PI hydrolysis. The potentiation of the physiological ADP by concurrent Group I/II mGluR activation described here strongly endorses the idea of amplification in IP_3 production, resulting in the downstream increase in intracellular calcium release and the enhanced activation of the calcium

dependent ADP conductances, finally resulting in a larger amplitude mGluR-mediated ADP.

The finding that physiological responses mediated by Group I mGluRs can be potentiated with concurrent Group II mGluR activation could influence the interpretation of previously published data on mGluR properties. For example, PI hydrolysis mediated by excitatory amino acids declines progressively during postnatal development (Schoepp and Hillman 1990) and there is a strong correlation between PI hydrolysis and mGluR5 expression in the brain (Casabona et al. 1997). They suggest that this observation may be due to a less efficient coupling of mGluR1 to PI hydrolysis compared to mGluR5. Considering the observations described here, an alternative hypothesis may be that mGluR subtypes (mGluR1, mGluR5, or both) are more efficiently coupled to Group II potentiation of their IP_3 response in early postnatal development. Future experiments correlating mGluR subtype-specific drugs, varied subtype expression levels at different ages, and relative potentiation of each mGluR subtype by concurrent Group II activation would contribute to the current findings and provide a clearer explanation about relative contribution from each mGluR subtype to the ADP response.

The observation of a previously subthreshold ADP response potentiated to above spike threshold by Group II mGluR activation (see Figure 18 and 19) suggests a potentially important clinical role for this mechanism. Based on the reported specific role of both mGluR1 and mGluR5 subtypes in excitotoxic responses in the brain (Bruno et al. 1995; Mukhin et al. 1996; Strasser et al. 1998; Bruno et al. 1999; Bruno et al. 2000) this

information may be important for targeting pharmaceutical development and eventual clinical application.

Specificity of the mGluR Potentiation

The present research demonstrated that the spike-dependent mAChR-mediated ADP in neocortical neurons is also dependent on release of calcium from IP_3R-sensitve stores. In this case one might expect that concurrent Group II mGluR activation would potentiate the mAChR-mediated response as well. Previous biochemical studies had not been able to demonstrate a Group II mGluR-mediated potentiation of the mAChR-induced PI hydrolysis (Nicoletti et al. 1993; Mistry et al. 1998), however, although there are reports of convergence of the metabolic pathways activated by mGluRs and mAChRs (Guerineau et al. 1995).

In accordance with the biochemical studies, I found no physiologic amplification of the carbachol-evoked ADP with concurrent Group II mGluR activation, indicating that a specific interaction between the Group I and Group II mGluRs is required for the amplification of the mGluR-dependent ADP in the neocortex. The present data does not exclude the possibility that the conductance underlying the mGluR- and mAChR-mediated ADP in neocortical neurons is the same, but that the potentiation of this conductance by Group II mGluR activation is unique to Group I mGluR activation. Based on this specificity, the mechanism of potentiation probably occurs near the receptor itself, and is not simply the result of a gross Group II mGluR-mediated modulation of PLC or other downstream intracellular messengers.

Further Exploration of Group II Potentiation

The fact that Group II mGluR activation could potentiate the ADP to suprathreshold level and cause repetitive firing offers potential clinical interest. Research of the Group II mGluRs has focused on neuroprotective properties by the activation of presynaptic Group II mGluRs (Bruno et al. 1994; Anwyl 1999). Obviously in this case, the effect of Group II mGluR activation is postsynaptic, and would appear far from neuroprotective. An interesting experiment would be to test a Group II mGluR antagonist against an ADP evoked using ACPD. If a suprathreshold ADP were evoked with either ACPD or combined Group I and II agonists, would the application of Group II antagonist bring the ADP below threshold for repetitive firing by inhibiting the potentiation?

Additional experiments are needed to further characterize the mGluR subtypes affected by Group II mGluR activation in neocortical neurons. Even though there is not a potent mGluR1 agonist currently available, the mGluR5 antagonist (MPEP) could be used to isolate the mGluR1-mediated component of the ADP and then see if the remaining portion could be potentiated with the addition of the Group II agonist, DCG-IV.

A calcium imaging study would be a valuable adjunct to our understanding of intracellular calcium levels during the mGluR-mediated potentiation. The experiment would monitor the changes in intracellular calcium concurrent with the potentiation. Potentially, this would provide an additional corollary between biochemical events and physiological results as well as provide additional physiological evidence supporting the role of IP_3R-mediated release of calcium in ADP generation.

The mechanism of Group II mGluR-mediated potentiation of the mGluR1- and/or mGluR5 receptor mediated PI hydrolysis remains merely conjecture at this point. A popular hypothesis suggests the possibility of the βγ subunit of the G-protein associated with Group II mGluRs activating the PLC-β isoenzymes in the Group I mGluR pathway (Smrcka and Sternweis 1993; Sternweis and Smrcka 1993; Wu et al. 1993a; Wu et al. 1993b). These studies describe specific binding sites on the PLC-β isoenzyme for the binding of α and βγ subunits of G-proteins. Others refute this conclusion however (Mistry et al. 1998), as activation of Group II mGluRs alone does not cause activation of the PI hydrolysis response. Further investigation of the mechanisms of the cross talk between the Group I and Group II mGluRs is required.

Summary

The present experiments have revealed the electrophysiological correlates in neocortical Layer 5 pyramidal neurons of several mGluR-mediated events reported in biochemical studies. The physiological response in neocortical neurons to evoked spikes during the stimulation of mGluRs is an ADP. Generation of the ADP depends on G-protein activation and on both [IP_3]- and [Ca^{2+}]-sensitive intracellular calcium stores. The mGluR-mediated ADP can also be evoked using high intensity synaptic stimulation, which is mediated in part by Group I subtype mGluR5. In general the ADP can be mediated by the activation of both mGluR1 and mGluR5 subtypes of Group I mGluRs. The activation of Group II mGluRs alone has no effect, but concurrent Group II mGluR activation potentiates the Group I-mediated ADP, part of which is due to the potentiation

of the Group I subtype mGlu5. The potentiation of the Group I mGluR-mediated ADP appears specific to concurrent Group I/Group II mGluR activation, as Group II mGluR activation does not potentiate the carbachol-mediated ADP — even though carbachol reportedly uses the same intracellular pathway as Group I mGluRs. Future investigations will hopefully benefit from these results describing the metabotropic pathways involved in the generation of an afterdepolarization in Layer V pyramidal neurons.

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